Modification Form for Permit BIO-UWO-0256

Permit Holder: Lakshman Gunaratnam

PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOLOGICAL AGENTS.
PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOLOGICAL AGENTS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF.

Approved Personnel
(Please stroke out any personnel to be removed)

Rushi Ghandi

Phoebe Zhong

April Huang

Sahra Nathoo

Ola Ismail

Xinghong Xhang

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biological Agent(s) to be removed	Write additional Biological Agent(s) for approval below. Give the full name
Approved Microorganisms	E.coli, Lentivirus	
Approved Primary and Established Cells	Mouse[primary] kidney, spleen, blood, bone marrow, CT26.WT. Human[established]786-0, 769-P, HEK293, HEK293T/17, Jurkat, HK-2 Rodent[established] JAWSII, mIMCD-3, CMT-93, DC. 2.4. Porcine [established]LLC-	
Approved Use of Human Source Material		
Approved Genetic Modifications (Plasmids/Vectors)	[plasmids]: pcDNA3. [vectors]: pLUX-puro proprietory	pCMV 'delta R8.2 pCOH ? VSVG ?
Approved Use of Animals	Musculus	
Approved Biological Toxin(s)	C3 Exotoxin, Phalloidin, SB202190, (S)-(+)-Camptothecin, GM6001, Gemcitabine, Tunicamycin, cylcoheximide, MG132, ALLN, Y27632, Dithiotheitol, Bafilomyun, Pharbol-12, TAPI-O, poxorubicin, oxaliplatin	

Approved Gene Therapy		
Approved Plants and Insects		

As the Principal Investigator, I have ensured that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/newposition.htm

Signature of Permit Holder: _____

Current Classification: 2+ **Containment Level for Added Biohazards:** _____

Date of Last Biohazardous Agents Registry Form: Jun 29, 2010

Date of Last Modification (if applicable): Feb 17, 2012

BioSafety Officer(s)*: _____

***For work being performed at Institutions affiliated with Western University, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to Western University Biosafety Officer.**

Chair, Biohazards Subcommittee: **Date:**

Biosafety_ModificationSummary_2012_June_11

The purpose for adding these agents to the protocol is as follows: We are generating mouse and human cell lines either expressing the KIM-1 transgene or shRNA knockdown for endogenous KIM-1 in the respective species. We will be doing this by direct DNA transfection or by using Lentivirus infection. Once infected/transfected, the cells will be grown in selection medium for a few weeks and positive cells will be used as stable cell lines. These cells will then be injected into mice (not with virus) to test their tumorigenic potential in NUDE or wild-type mice as per our animal protocol. These are replication defective viruses and should not be sheding virus. We will not introduce any virus into any animals.

Western University
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: April 13, 2012
Biosafety Website: www.uwo.ca/humanresources/biosafety/

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☒ YES ☐ NO If **NO**, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☒ YES, complete table below ☐ NO

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transformed or Transfected	Will there be a change due to transformation of the bacteria?	Will there be a change in the pathogenicity of the bacteria after the genetic modification?	What are the consequences due to the transformation of the bacteria?	If plasmids are being used to transfect cells what is the consequence on the eukaryotic cells?
E.coli							
DH5a	**pcDNA3**		**TIM-1**				**not transforming**

**** Please attach a Material Safety Data Sheet or equivalent if available.***
***** Please attach a plasmid map.***
******No Material Safety Data Sheet is required for the following strains of E. coli:***
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

4.3 Will genetic modification(s) of bacteria and/or cells involving viral vectors be made?
☒ YES, complete table below ☐ NO

Virus/Plasmid Used for Vector Construction	Vector(s) *	Source of Vector/Plasmid	Gene(s) Transduced/ Transfected	Describe the change that results from transduction/transfection
	pcDH Proprietory vector from Santa Cruz	**ADDGENE Santa Cruz Biotech**	**TIM-1**	**Not transforming**

**** Please attach a Material Safety Data Sheet or equivalent.***

4.3.1 Will virus be replication defective? ☒ YES ☐ NO

4.3.2 Will virus be infectious to humans or animals? ☐ YES ☒ NO

4.3.3 Will this be expected to increase the containment level required? ☐ YES ☒ NO

5.0 Will genetic sequences from the following be involved?

- HIV ☐ NO ☒ YES, specify
- HTLV 1 or 2 or genes ☒ NO ☐ YES, specify
- SV 40 Large T antigen ☒ NO ☐ YES
- E1A oncogene ☒ NO ☐ YES
- Known oncogenes ☒ NO ☐ YES, specify
- Other human or animal pathogen and or their toxins ☒ NO ☐ YES, specify

5.1 Is any work being conducted with prions or prion sequences? ☒ NO ☐ YES

Additional Comments: **Animals will be injected with cells transduced with Virus after culture and selection, no virus replication should occur**

8.0 Animal Experiments

8.1 Will live animals be used? ☒ YES ☐ NO If **NO**, please proceed to section 9.0

8.2 List animal species to be used: **MOUSE**

8.3 AUS protocol number(s): **2010-230**

8.4 List the location(s) for the animal experimentation and housing: **Health Sciences**

8.5 Will any of the agents listed in Sections 1-7 be used in live animals
☒ NO ☐ YES, specify:

8.6 Will the agent(s) be shed by the animal:
☐ YES ☒ NO, please justify:

8.7 Indicate the PHAC or CFIA containment level used: ☐ 1 ☐ 2 ☒ 2+ ☐ 3

9.0 Use of Animal species with Zoonotic Hazards

9.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? ☐ YES ☒ NO - If **NO**, please proceed to section 10.0

9.2 Will live animals be used? ☐ YES ☐ NO

9.3 If **YES**, please specify the animal(s) used:

♦ Pound source dogs	☐ YES	☐ NO
♦ Pound source cats	☐ YES	☐ NO
♦ Cattle, sheep or goats	☐ YES, species	☐ NO
♦ Non-human primates	☐ YES, species	☐ NO
♦ Wild caught animals	☐ YES, species & colony #	☐ NO
♦ Birds	☐ YES, species	☐ NO
♦ Amphibians	☐ YES, species	☐ NO
♦ Others (wild or domestic)	☐ YES, specify	☐ NO

9.4 If no live animals are used, please specify the source of the specimens:

addgene
A better way to share plasmids

Browse > Didier Trono > Trono Lab Packaging and Envelo... > pCMV delta R8.2

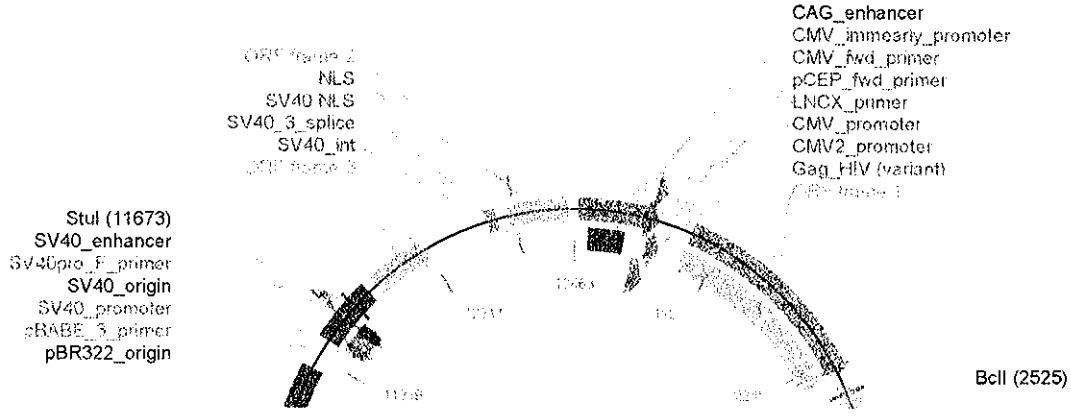
Plasmid 12263: pCMV delta R8.2

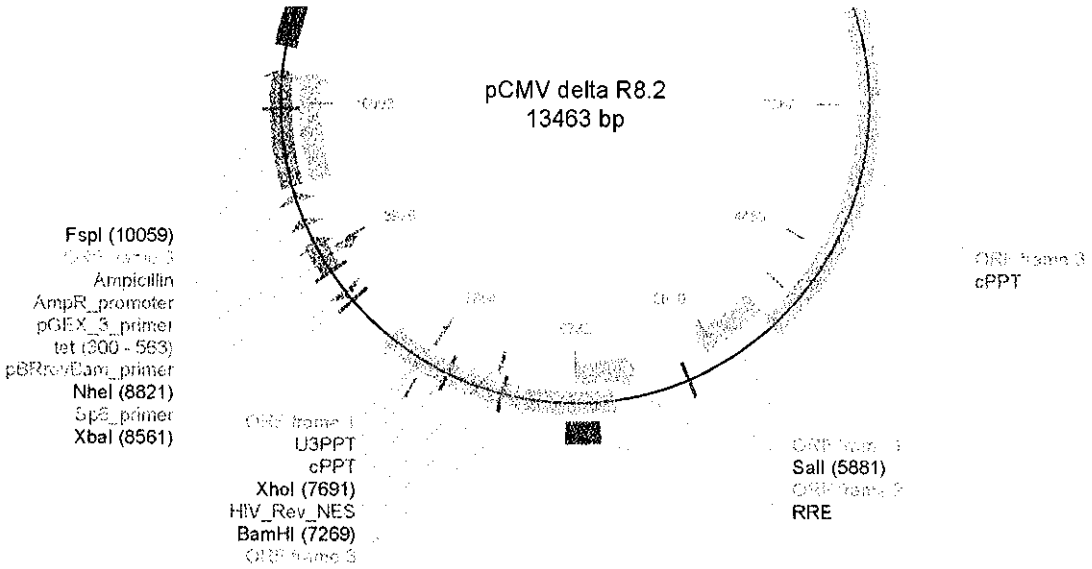
Gene/insert name:	HIV-1 GAG/POL, Tat and Rev
Vector backbone:	pCMVR8.2 (Search Vector Database)
Vector type:	Mammalian Expression, Lentiviral
Vector type:	Packaging
Backbone size w/o insert:	8128
5' sequencing primer:	CMV forward List of Sequencing Primers
Bacterial resistance(s)	Ampicillin
Growth strain(s)	DH5alpha
Growth temperature (°C):	37
High or low copy:	High Copy
Sequence:	View sequences (2)
Supplemental document:	Digest Plasmid 12263 (application/pdf)
Principal Investigator:	Didier Trono
Terms and Licenses:	MTA

Comments: Packaging plasmid.

Please note that the full sequence for this plasmid is approximated and not fully verified. Please visit the Trono lab http://tronolab.epfl.ch for cloning strategies, protocols, publications, and more. See LentiWeb http://www.lentiweb.com for discussion on cloning strategies and protocols.

Addgene has sequenced a portion of this plasmid for verification. Click here for the sequencing result.





Feature Name	Start	End
CMV_immearly_promoter	27	603
CAG_enhancer	106	393
CMV_fwd_primer	560	580
CMV_promoter	561	630
CMV2_promoter	573	692
pCEP_fwd_primer	604	623
LNCX_primer	606	630
Gag_HIV (variant)	880	2388
cPPT	4881	4896
RRE	6563	6796
HIV_Rev_NES	7319	7348
cPPT	7863	7878
U3PPT	7863	7884
Sp6_primer	8600	8583
tet (300 - 563)	8839	9102
pBRrevBam_primer	8910	8891
pGEX_3_primer	9244	9266
AmpR_promoter	9425	9453
Ampicillin	9495	10355
pBR322_origin	10510	11129
pBABE_3_primer	11375	11355
SV40_enhancer	11814	11361
SV40_promoter	11373	11641
SV40_origin	11540	11617
SV40pro_F_primer	11602	11621
SV40_int	12823	12838
SV40_3_splice	12844	12891
SV40 NLS	13019	13039
NLS	13025	13039

ORF	Start	End
ORF frame 1	880	2388

ORF frame 3	2454	5192
ORF frame 1	5137	5715
ORF frame 2	6736	6233
ORF frame 3	6441	7589
ORF frame 1	7591	8211
ORF frame 3	9495	10355
ORF frame 3	11889	12347
ORF frame 2	12968	13441

Enzyme Name	**Cut**
BclI	2525
SalI	5881
BamHI	7269
XhoI	7691
XbaI	8561
NheI	8821
FspI	10059
StuI	11673

Please acknowledge the principal investigator and cite this article if you use this plasmid in a publication. Also, please include the text "Addgene plasmid 12263" in your Materials and Methods section.

I. Introduction and Background

A. Purpose of this Manual

This manual provides information describing how to package lentivector expression constructs in pseudoviral particles and use packaged expression constructs for transduction of target cells. Specifically, it provides critical instructions on how to package an HIV-based or FIV-based Lentivector Expression construct in VSV-G pseudotyped viral particles by co-transfecting 293TN Producer Cells with a Lentivector Expression construct and the pPACKH1™ (for HIV-based constructs) or pPACKF1™ (for FIV-based constructs) Packaging Plasmid Mix. Recommendations are also provided for selection and use of HIV-based and FIV-based lentivector systems for transducing a wide range of target cells.

This manual does not include information about construction of expression constructs in lentiviral expression vectors. Information on making constructs using these vectors is available in the user manuals for each of SBI's Lentivector Cloning and Expression Vectors. User manuals, which are provided with each of the Lentivector products, can also be accessed on the SBI website (http://www.systembio.com). Before using the reagents and material supplied with this product, please read the entire user manual.

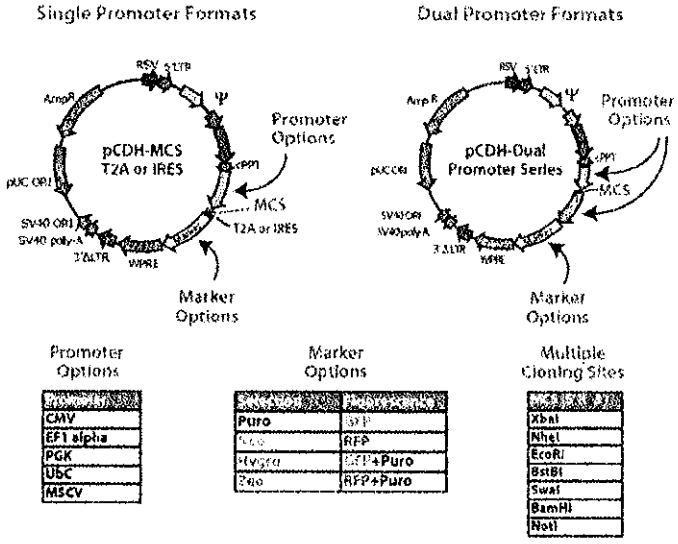
B. Lentiviral Expression Systems

Lentiviral expression vectors are the most effective vehicles for transducing and stably expressing different effector molecules (siRNA, cDNA, DNA fragments, antisense, ribozymes, etc.) or reporter constructs in almost any mammalian cell,including non-dividing cells and whole model organisms (Cann, 2000). As with standard plasmid vectors, it is possible to introduce lentiviral constructs in plasmid form into the cells with low-to-medium efficiency and get transient expression of effectors (reporters) using conventional transfection protocols. By packaging the lentiviral expression construct into pseudoviral particles, you can obtain highly efficient transduction (up to 100%), even with the most difficult to transfect cells, such as primary, stem, and differentiated cells.

The expression construct transduced in cells is integrated into genomic DNA and provides stable, long-term expression of siRNA, cDNA or reporter gene. Endogenously expressed siRNA effectors provide long-term silencing of the target gene and allow the researcher to generate cell lines and transgenic organisms with a stable knockdown phenotype for functional studies. Expression of full-length cDNAs from integrated viral constructs is a unique tool to study gain-of-function effect for cellular phenotypes. Stably integrated transcriptional reporter constructs are a novel approach to the study of transcriptional regulation in the natural chromosomal environment and the monitoring of specific signaling pathways. Moreover, lentiviral delivery does not produce the non-

Hybrid RSV-5'LTR promoter	For HIV-based vectors. Provides a high level of expression of full-length pseudoviral constructs in 293 producer cells.
Hybrid CMV-5'LTR promoter	For FIV-based vectors. Provides a high level of expression of full-length pseudoviral constructs in 293 producer cells
cPPT, GAG, LTRs	Genetic elements necessary for the packaging, transduction, and stable integration of the viral expression construct into genomic DNA
SV40 origin	Provides stable propagation of the lentiviral plasmid in 293 producer cells.
pUC origin	Ensures high copy replication and maintenance of the plasmid in *E.coli* cells
Ampicillin resistance	Used for selection in *E. coli* cells.
WPRE element	Enhances stability and translation of the lentivector-driven transcripts
SV40 polyadenylation signal	Enables efficient termination of transcription and processing of recombinant transcripts.

SBI offers a variety of promoter and reporter options, including GFP, RFP, Puromycin, Hygromycin, Neomycin and Zeocin selection, as well as inducible expression vectors. All SBI lentivectors contain viral stability elements, such as cPPT, WPRE and RRE sequences, for enhanced packaging and infection efficiency.



Modification Form for Permit BIO-UWO-0256

Permit Holder: Lakshman Gunaratnam

Approved Personnel
(Please stroke out any personnel to be removed)

Rushi Ghandi

Phoebe Zhong

April Huang

Sahra Nathoo

Ola Ismail

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Additional Personnel
(Please list additional personnel here)

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Approved Primary and Established Cells	Mouse[primary] kidney, spleen, blood, bone marrow. Human[established]786-0, 769-P, HEK293, HEK293T/17, Jurkat, HK-2 Rodent[established] JAWSII, mIMCD-3, CMT-93, DC. 2.4. Porcine [established]LLC-PK1,	CT26.WT
Approved Use of Human Source Material		
Approved Genetic Modifications (Plasmids/Vectors)	[plasmids]: pcDNA3. [vectors]: pLUX-puro proprietory	
Approved Use of Animals	Musculus	
Approved Biological Toxin(s)	C3 Exotoxin, Phalloidin, SB202190, (S)-(+)-Camptothecin, GM6001, Gemcitabine, Tunicamycin, cylcoheximide, MG132, ALLN, Y27632, Dithiotheitol, Bafilomyun, Pharbol-12, TAPI-O	Doxorubicin Oxaliplatin
Approved Gene Therapy		

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF..*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: _____ Jan 17, 2012

Current Classification: 2+ **Containment Level for Added Biohazards:** _____

Date of Last Biohazardous Agents Registry Form: Jun 29, 2010

Date of Last Modification (if applicable): Nov 24, 2011

BioSafety Officer(s): Jstanley Feb 16, 2012

Chair, Biohazards Subcommittee: _____ **Date:** 17/4

Doxorubicin: LD50 = ~698 mg/kg (mouse) - oral
Max single use = 20 µM × 10 ml
Max storage = 10 MG
Sigma

Oxaliplatin: LD 50 (IP) = 14.3 mg/kg
Max storage = 5MG
Max single use = 5 mg/ml × 10 ml
Sigma

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Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Canada, China, Hong Kong, India, Israel, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Print this Page

Cell Biology

ATCC® Number:	**CRL-2638™** Order this Item	**Price:**	**$329.00**
Designations:	CT26.WT		
Depositors:	N Restifo		
Biosafety Level:	1		
Shipped:	frozen		
Medium & Serum:	See Propagation		
Growth Properties:	adherent		
Organism:	*Mus musculus* deposited as mouse		
Morphology:	fibroblast		
Source:	**Organ:** colon **Strain:** BALB/c **Disease:** carcinoma		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Applications:	CT26 is an N-nitroso-N-methylurethane-(NNMU) induced, undifferentiated colon carcinoma cell line. The cell line can be used with CT26.CL25 (ATCC CRL-2639) as a model for testing immunotherapy protocols and in studies on the host immune response.		
Tumorigenic:	Yes		
Antigen Expression:	H-2d [53315]		
Comments:	CT26 is an N-nitroso-N-methylurethane-(NNMU) induced, undifferentiated colon carcinoma cell line. It was cloned to generate the cell line designated CT26.WT (ATCC CRL-2638). CT26.WT was stably transduced with the retroviral vector LXSN that contains the lacZ gene encoding the model tumor associated antigen (TAA), beta-galactosidase (beta-gal) to obtain the lethal subclone CT26.CL25 (ATCC CRL-2639). The growth rate and lethality of CT26.CL25 and CT26.WT is virtually identical despite the expression by CT26.CL25 of the model TAA, beta-galactosidase, in normal mice. The cell line can be used with CT26.CL25 (ATCC CRL-2639) as a model for testing immunotherapy protocols and in studies on the host immune		

Related Links

- NCBI Entrez Search
- Make a Deposit
- Frequently Asked Questions
- Material Transfer Agreement
- Technical Support
- Related Cell Culture Products

Login Required

- Product Information Sheet

response.
A culture submitted to the ATCC in July of 2001 was found to be contaminated with mycoplasma. Progeny were cured by a 21-day treatment with BM Cycline.
The cells were assayed for mycoplasma, by the Hoechst stain, PCR and the standard culture test, after a six-week period following treatment. All tests were negative.

Propagation:

ATCC complete growth medium: The base medium for this cell line is ATCC-formulated RPMI-1640 Medium, Catalog No. 30-2001. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Atmosphere: air, 95%; carbon dioxide (CO_2), 5%
Temperature: 37.0°C

Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:10 is recommended
Medium Renewal: Every 2 to 3 days

Preservation:

Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2001
recommended serum:ATCC 30-2020
derivative:ATCC CRL-2639

References:

53315: Wang M, et al. Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. J. Immunol. 154: 4685-4692, 1995. PubMed: 7722321

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Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Doxorubicin
Proposed Use Dose:	200 µg
Proposed Storage Dose:	10000 µg
LD_{50} (species):	698000 µg/kg

Calculation:

698000 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 34900000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = 3490000

Comments/Recommendations: No LD50 data found.

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Oxaliplatin
Proposed Use Dose:	50 µg
Proposed Storage Dose:	5000 µg
LD_{50} (species):	14300 µg

Calculation:

14300 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 715000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = 71500

Comments/Recommendations:

Health	2
Fire	1
Reactivity	0
Personal Protection	E

Material Safety Data Sheet
Doxorubicin hydrochloride MSDS

Section 1: Chemical Product and Company Identification

Product Name: Doxorubicin hydrochloride

Catalog Codes: SLD2740

CAS#: 25316-40-9

RTECS: QI9295900

TSCA: TSCA 8(b) inventory: No products were found.

CI#: Not available.

Synonym: 10-[(3)-Amino-2,3,6-trideoxy-alpha-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione (8S-cis)hydrochloride

Chemical Name: Not available.

Chemical Formula: C27H30ClNO11

Contact Information:

Sciencelab.com, Inc.
14025 Smith Rd.
Houston, Texas 77396

US Sales: **1-800-901-7247**
International Sales: **1-281-441-4400**

Order Online: ScienceLab.com

CHEMTREC (24HR Emergency Telephone), call: 1-800-424-9300

International CHEMTREC, call: 1-703-527-3887

For non-emergency assistance, call: 1-281-441-4400

Section 2: Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Doxorubicin hydrochloride	25316-40-9	100

Toxicological Data on Ingredients: Doxorubicin hydrochloride: ORAL (LD50): Acute: 698 mg/kg [Mouse].

Section 3: Hazards Identification

Potential Acute Health Effects:
Very hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, of inhalation (lung sensitizer). Hazardous in case of inhalation (lung irritant). Slightly hazardous in case of skin contact (corrosive, permeator). Inflammation of the eye is characterized by redness, watering, and itching. Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering.

Potential Chronic Health Effects:
CARCINOGENIC EFFECTS: Classified 2 (Reasonably anticipated.) by NTP. 3 (Not classifiable for human.) by IARC. MUTAGENIC EFFECTS: Not available. TERATOGENIC EFFECTS: Not available. DEVELOPMENTAL TOXICITY: Not available. Repeated or prolonged exposure is not known to aggravate medical condition.

Section 4: First Aid Measures

Eye Contact:
Check for and remove any contact lenses. Immediately flush eyes with running water for at least 15 minutes, keeping eyelids open. Cold water may be used. Do not use an eye ointment. Seek medical attention.

Skin Contact:
After contact with skin, wash immediately with plenty of water. Gently and thoroughly wash the contaminated skin with running water and non-abrasive soap. Be particularly careful to clean folds, crevices, creases and groin. Cold water may be used. Cover the irritated skin with an emollient. If irritation persists, seek medical attention. Wash contaminated clothing before reusing.

Serious Skin Contact:
Wash with a disinfectant soap and cover the contaminated skin with an anti-bacterial cream. Seek immediate medical attention.

Inhalation: Allow the victim to rest in a well ventilated area. Seek immediate medical attention.

Serious Inhalation: Not available.

Ingestion:
Do not induce vomiting. Examine the lips and mouth to ascertain whether the tissues are damaged, a possible indication that the toxic material was ingested; the absence of such signs, however, is not conclusive. Loosen tight clothing such as a collar, tie, belt or waistband. If the victim is not breathing, perform mouth-to-mouth resuscitation. Seek immediate medical attention.

Serious Ingestion: Not available.

Section 5: Fire and Explosion Data

Flammability of the Product: May be combustible at high temperature.

Auto-Ignition Temperature: Not available.

Flash Points: Not available.

Flammable Limits: Not available.

Products of Combustion: These products are carbon oxides (CO, CO2), nitrogen oxides (NO, NO2...), halogenated compounds.

Fire Hazards in Presence of Various Substances: Not available.

Explosion Hazards in Presence of Various Substances:
Risks of explosion of the product in presence of mechanical impact: Not available. Risks of explosion of the product in presence of static discharge: Not available.

Fire Fighting Media and Instructions:
SMALL FIRE: Use DRY chemical powder. LARGE FIRE: Use water spray, fog or foam. Do not use water jet.

Special Remarks on Fire Hazards: Not available.

Special Remarks on Explosion Hazards: Not available.

Section 6: Accidental Release Measures

Small Spill:
Use appropriate tools to put the spilled solid in a convenient waste disposal container. Finish cleaning by spreading water on the contaminated surface and dispose of according to local and regional authority requirements.

Large Spill:
Use a shovel to put the material into a convenient waste disposal container. Finish cleaning by spreading water on the contaminated surface and allow to evacuate through the sanitary system.

Section 7: Handling and Storage

Precautions:
Keep container dry. Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not ingest. Do not breathe dust. Never add water to this product In case of insufficient ventilation, wear suitable respiratory equipment If ingested, seek medical advice immediately and show the container or the label. Avoid contact with skin and eyes

Storage:
Keep container dry. Keep in a cool place. Ground all equipment containing material. Carcinogenic, teratogenic or mutagenic materials should be stored in a separate locked safety storage cabinet or room.

Section 8: Exposure Controls/Personal Protection

Engineering Controls:
Use process enclosures, local exhaust ventilation, or other engineering controls to keep airborne levels below recommended exposure limits. If user operations generate dust, fume or mist, use ventilation to keep exposure to airborne contaminants below the exposure limit.

Personal Protection:
Splash goggles. Lab coat. Dust respirator. Be sure to use an approved/certified respirator or equivalent. Gloves.

Personal Protection in Case of a Large Spill:
Splash goggles. Full suit. Dust respirator. Boots. Gloves. A self contained breathing apparatus should be used to avoid inhalation of the product. Suggested protective clothing might not be sufficient; consult a specialist BEFORE handling this product.

Exposure Limits: Not available.

Section 9: Physical and Chemical Properties

Physical state and appearance: Solid. (Solid crystalline powder.)

Odor: Not available.

Taste: Not available.

Molecular Weight: 579.99 g/mole

Color: Yellow to red.

pH (1% soln/water): Not available.

Boiling Point: Not available.

Melting Point: Decomposes. (204.5°C or 400.1°F)

Critical Temperature: Not available.

Specific Gravity: Not available.

Vapor Pressure: Not applicable.

Vapor Density: Not available.

Volatility: Not available.

Odor Threshold: Not available.

Water/Oil Dist. Coeff.: Not available.

Ionicity (in Water): Not available.

Dispersion Properties: See solubility in water.

Solubility: Soluble in cold water.

Section 10: Stability and Reactivity Data

Stability: The product is stable.

Instability Temperature: Not available.

Conditions of Instability: Not available.

Incompatibility with various substances: Not available.

Corrosivity: Non-corrosive in presence of glass.

Special Remarks on Reactivity: Not available.

Special Remarks on Corrosivity: Not available.

Polymerization: No.

Section 11: Toxicological Information

Routes of Entry: Eye contact. Inhalation. Ingestion.

Toxicity to Animals: Acute oral toxicity (LD50): 698 mg/kg [Mouse].

Chronic Effects on Humans:
CARCINOGENIC EFFECTS: Classified 2 (Reasonably anticipated.) by NTP. 3 (Not classifiable for human.) by IARC.

Other Toxic Effects on Humans:
Very hazardous in case of skin contact (irritant), of ingestion, of inhalation (lung sensitizer). Hazardous in case of inhalation (lung irritant). Slightly hazardous in case of skin contact (corrosive, permeator).

Special Remarks on Toxicity to Animals: Not available.

Special Remarks on Chronic Effects on Humans: Risk of spontaneous abortion in animal.

Special Remarks on other Toxic Effects on Humans: Not available.

Section 12: Ecological Information

Ecotoxicity: Not available.

BOD5 and COD: Not available.

Products of Biodegradation:
Possibly hazardous short term degradation products are not likely. However, long term degradation products may arise.

Toxicity of the Products of Biodegradation: The products of degradation are more toxic.

Special Remarks on the Products of Biodegradation: Not available.

Section 13: Disposal Considerations

Waste Disposal:

Section 14: Transport Information

DOT Classification: Not a DOT controlled material (United States).

Identification: Not applicable.

Special Provisions for Transport: Not applicable.

Section 15: Other Regulatory Information

Federal and State Regulations:
Pennsylvania RTK: Doxorubicin hydrochloride TSCA 8(b) inventory: No products were found.

Other Regulations: OSHA: Hazardous by definition of Hazard Communication Standard (29 CFR 1910.1200).

Other Classifications:

WHMIS (Canada): CLASS D-2A: Material causing other toxic effects (VERY TOXIC).

DSCL (EEC):
R37/38- Irritating to respiratory system and skin. R41- Risk of serious damage to eyes. R42- May cause sensitization by inhalation. R45- May cause cancer.

HMIS (U.S.A.):

Health Hazard: 2

Fire Hazard: 1

Reactivity: 0

Personal Protection: E

National Fire Protection Association (U.S.A.):

Health: 2

Flammability: 1

Reactivity: 0

Specific hazard:

Protective Equipment:
Gloves. Lab coat. Dust respirator. Be sure to use an approved/certified respirator or equivalent. Wear appropriate respirator when ventilation is inadequate. Splash goggles.

Section 16: Other Information

References: Not available.

Other Special Considerations: Not available.

Created: 10/11/2005 11:52 AM

Last Updated: 11/01/2010 12:00 PM

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Material Safety Data Sheet

Version 4.5
Revision Date 04/07/2011
Print Date 10/27/2011

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : Oxaliplatin

Product Number : O9512
Brand : Sigma
Product Use : For laboratory research purposes.

Supplier : Sigma-Aldrich Canada, Ltd
2149 Winston Park Drive
OAKVILLE ON L6H 6J8
CANADA

Manufacturer : Sigma-Aldrich Corporation
3050 Spruce St.
St. Louis, Missouri 63103
USA

Telephone : +1 9058299500
Fax : +1 9058299292
Emergency Phone # (For both supplier and manufacturer) : 1-800-424-9300
Preparation Information : Sigma-Aldrich Corporation
Product Safety - Americas Region
1-800-521-8956

2. HAZARDS IDENTIFICATION

Emergency Overview

Target Organs

Liver injury may occur., Kidney, ears, Blood, Peripheral nervous system., Bone marrow, Testes., Female reproductive system.

WHMIS Classification

D2B Toxic Material Causing Other Toxic Effects

Moderate skin irritant
Moderate respiratory irritant
Moderate eye irritant
Skin sensitiser

GHS Classification

Skin irritation (Category 2)
Eye irritation (Category 2A)
Skin sensitization (Category 1)
Carcinogenicity (Category 2)
Specific target organ toxicity - single exposure (Category 3)

GHS Label elements, including precautionary statements

Pictogram

Signal word Warning

Hazard statement(s)

H315	Causes skin irritation.
H317	May cause an allergic skin reaction.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.
H351	Suspected of causing cancer.

Precautionary statement(s)

P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.

P280	Wear protective gloves.
P305 + P351 + P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

HMIS Classification

Health hazard:	2
Chronic Health Hazard:	*
Flammability:	0
Physical hazards:	0

Potential Health Effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.
Ingestion	May be harmful if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : [SP-4-2-(1R-trans)]-(1,2-Cyclohexanediamine-N,N')[ethanedioata(2--)-O,O']platinum

Formula : $C_8H_{14}N_2O_4Pt$ $C_8H_{14}N_2O_4Pt$

Molecular Weight : 397.29 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
Oxaliplatin			
61825-94-3	-	-	-

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Consult a physician.

In case of eye contact
Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIRE-FIGHTING MEASURES

Conditions of flammability
Not flammable or combustible.

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)

Explosion data - sensitivity to mechanical impact
no data available

Explosion data - sensitivity to static discharge
no data available

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use personal protective equipment. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Avoid breathing dust.

Environmental precautions
Prevent further leakage or spillage if safe to do so. Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: 2 - 8 °C

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
Where risk assessment shows air-purifying respirators are appropriate use a full-face particle respirator type N100 (US) or type P3 (EN 143) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Face shield and safety glasses Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
Complete suit protecting against chemicals, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

Specific engineering controls
Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid
Colour	no data available

Safety data

pH	no data available
Melting point/freezing point	no data available

Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available
Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)
Other decomposition products - no data available

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
Inhalation LC50
Dermal LD50
no data available

Other information on acute toxicity
LD50 Intraperitoneal - rat - 14.3 mg/kg

LD50 Intraperitoneal - mouse - 19.8 mg/kg

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization
May cause allergic skin reaction.

Germ cell mutagenicity

no data available

Carcinogenicity

This product is or contains a component that is not classifiable as to its carcinogenicity based on its IARC, ACGIH, NTP, or EPA classification.

Limited evidence of carcinogenicity in animal studies

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
Inhalation - May cause respiratory irritation.

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Ingestion	May be harmful if swallowed.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.

Signs and Symptoms of Exposure
bone marrow depression, Liver injury may occur.

Synergistic effects
no data available

Additional Information
RTECS: TP2275850

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

WHMIS Classification

D2B	Toxic Material Causing Other Toxic Effects	Moderate skin irritant Moderate respiratory irritant Moderate eye irritant Skin sensitiser

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Modification Form for Permit BIO-UWO-0256

Permit Holder: Lakshman Gunaratnam

Approved Personnel
(Please stroke out any personnel to be removed)

~~Ola Ismail~~

Xinghong Xhang

Additional Personnel
(Please list additional personnel here)

*Sahra Nathoo
Xiaojin (April) Huang
Phoebe Zhong
Rushi Ghandi

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. Give the full name - do not abbreviate.
Approved Microorganisms	E.coli, Lentivirus	
Approved Primary and Established Cells	Mouse[primary] kidney, spleen, blood, bone marrow. Human[established]786-0, 769-P, HEK293, HEK293T/17, Jurkat, HK-2 Rodent[established] JAWSII, mIMCD-3, CMT-93 Porcine [established]LLC-PK1, canine	See attached: PC-3, OT-I, OT-II, ABI-HA, B16-OVa, EL4 ...
Approved Use of Human Source Material		
Approved Genetic Modifications (Plasmids/Vectors)	[plasmids]: pcDNA3. [vectors]: pLUX-puro proprietory	
Approved Use of Animals	Musculus	
Approved Biological Toxin(s)	C3 Exotoxin, Phalloidin	See attached: 1. Gemcitabine 2. Tunicamycin 3. Cycloheximide 4. MG132 5. ALLN 6. Y27632 7. Dithiothreitol 8. Bafilomycin 9. Pharbol-12 10. TAPI-0

* Per voicemail from PI, (last week) these students will work on this project.

* PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS
** PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF.

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: _____ Aug 25, 2011

Current Classification: 2+ **Containment Level for Added Biohazards:** _____

Date of Last Biohazardous Agents Registry Form: Jun 29, 2010

Date of Last Modification (if applicable): _____

BioSafety Officer(s): NW 21/11 _____

Chair, Biohazards Subcommittee: _____ **Date:** 24 Nov 2011

Dr. Lakshman Gunaratnam BIO-UWO-0256
Proposed Changes as of Aug 25, 2011

Proposed use of agents:

Gemcitabine: Induction of apoptosis *in vitro* and *in vivo in* AB1-HA-GR/AB1-HA cells

Tunicamycin: Induction of ER stress in cells; LD50: >0.4mg/kg; single use 5 micrograms; storage 5mg

Cycloheximide: Used to inhibit protein translation in cells; LD50 2mg/kg; single use 100 micrograms ; storage 1 gram

MG132: Inhibition of proteasome in cells' LD50 N/A; single use 1 microgram; storage 1 milligram

ALLN: Inhibition of proteasome in cells; LD50 N/A; single use 5 micrograms; storage 5 milligrams

Y27632: Highly potent, cell permeable, selective and ATP competitive inhibitor of ROCK1 and ROCK2 (IC_{50}=800nM). Used to inhibit Rho Kinase in cells *in* vitro; LD50 N/A; single use 1 microgram; storage 1mg.

Dithiotheitol (DTT): Ruducing agent in lysis buffers; LD50 400mg/kg; single use 1 microgram; storage 5 grams

Bafilomycin A1: Inhibition of lysosomal function in cells; LD50 N/A; single use 1 microgram; storage 100 micrograms

Phorbol-12-myristate-13-acetate (PMA): trigger ectodomain cleavage of proteins via MMP; LD50 N/A; sing le use 0.1-1 microgram; storage 1 milligram

TAPI-0: trigger ectodomain cleavage of proteins via MMP; LD50 N/A; single use 1 microgram; storage 1 milligram

Proposed use of cell lines:

PC-3: Human Prostate cancer cell line used for testing KIM-1 phosphorylation in cell culture only.

OT-I: T cell receptor transgenic T cell line used for testing cross-presentation in cell culture. These cells are derived from the OT-I T cell receptor transgenic mice (primary T cells). (see attached paper)

OT-II: T cell receptor transgenic T cell line used for testing cross-presentation in cell culture. These cells are derived from the OT-II T cell receptor transgenic mice (primary T cells).

All of the following cell lines will be used to establish in vivo tumours in mice (sub-cutaneous or intraperitoneal) and will be used in cell culture models to test antigen cross-presentation in the lab.

AB1-HA-GR: Gemcitabine-resistant mesothelioma cell line

AB1-HA: Gemcitabine-sesitive mesothelioma cell line

B16-Ova: Mouse melanoma cell line

B16-Ova-Bims: Mouse melanoma cell line modified to overexpress Bim

B16-Ova-FADD-DD: Mouse melanoma cell line modified to overexpress FADD

EL4: Mouse lymphoma cell line

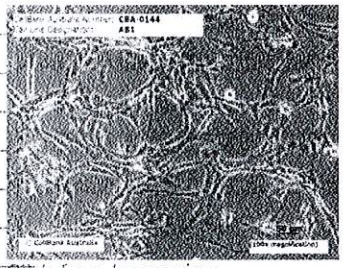
EL4-Ova: Mouse lymphoma cell line overexpressing OVA antigen

Info on Cell Line(s)

CellBank Australia

Children's Medical Research Institute

- Welcome
- Circulatory System Cell Lines
- Digestive System Cell Lines
- Endocrine System Cell Lines
- Immune System Cell Lines
- Integumentary System Cell Lines
- Lymphatic System Cell Lines
- Muscular-Skeletal System Cell Lines
- Nervous System Cell Lines
- Primary Cell Lines
- Reproductive System Cell Lines
- Respiratory System Cell Lines
- Urinary System Cell Lines
- STR Profiles
- Hybridomas
- Quote Cart



Click for a larger view

Product Group: AB1
AB1

Mouse malignant mesothelioma cell line

Basic Information Price Category D Catalogue number CBA0144 Other names Origin Mice (BALB/c strain, female, 6-8 weeks old) were exposed to crocidolite asbestos through intraperitoneal injection, resulting in tumour development. Cultures were established from malignant mesothelial cells obtained from ascites fluid. Features Cells are tumourigenic in syngeneic immunocompetent mice. Species Mouse Tissue Mesothelium Morphology Most cells are epithelial-like. AB12 and AB22 are stellate-shaped, while AB1 appears fibroblast-like but shows a typical epithelial "cobblestone" appearance when confluent. Disease Aesbestos induced mesothelioma Availability In stock - please confirm with CellBank Australia
Handling Information Risk group PC2 Known hazards GMO status N/A
Culture Information Preferred temperature 37 Preferred CO2 5 Preferred medium RPMI1640 + 5% FCS Passaging Optimal split ratio 1:8 Cryopreservation 10% DMSO + 90% FCS
References and Characteristics Markers Cells expressed Class-I, but not Class-II, H2 antigens. Virtually all cell lines expressed H-2Dd. Mutations Depositor Richard Lake - University of Western Australia Initial publication Davis MR, Manning LS, Whitaker D, Garlepp MJ, Robinson BW (1992) Establishment of a murine model of malignant mesothelioma. Int J Cancer 52: 881-886. Subsequent publications Cancer Immunol Immunother. 1994 Dec;39(6):347-59. Isogenic lines Species confirmation Confirmed as Mouse STR profile Not Available

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Induction of Tumor Cell Apoptosis In Vivo Increases Tumor Antigen Cross-Presentation, Cross-Priming Rather than Cross-Tolerizing Host Tumor-Specific CD8 T Cells[1]

Anna K. Nowak,* Richard A. Lake,* Amanda L. Marzo,[2]* Bernadette Scott,[3]* William R. Heath,† Edward J. Collins,‡ Jeffrey A. Frelinger,‡ and Bruce W. S. Robinson[4]*

Cross-presentation of cell-bound Ags from established, solid tumors to CD8 cells is efficient and likely to have a role in determining host response to tumor. A number of investigators have predicted that when tumor Ags are derived from apoptotic cells either no response, due to Ag "sequestration," or CD8 cross-tolerance would ensue. Because the crucial issue of whether this happens in vivo has never been addressed, we induced apoptosis of established hemagglutinin (HA)-transfected AB1 tumors in BALB/c mice using the apoptosis-inducing reagent gemcitabine. This shrank the tumor by ~80%. This induction of apoptosis increased cross-presentation of HA to CD8 cells yet neither gross deletion nor functional tolerance of HA-specific CD8 cells were observed, based on tetramer analysis, proliferation of specific CD8 T cells, and in vivo CTL activity. Interestingly, apoptosis primed the host for a strong antitumor response to a second, virus-generated HA-specific signal in that administration of an HA-expressing virus after gemcitabine administration markedly decreased tumor growth compared with viral administration without gemcitabine. Thus tumor cell apoptosis in vivo neither sequesters tumor Ags nor cross-tolerizes tumor-specific CD8 cells. This observation has fundamental consequences for the development of tumor immunotherapy protocols and for understanding T cell reactivity to tumors and the in vivo immune responses to apoptotic cells. *The Journal of Immunology,* **2003, 170: 4905–4913.**

Antigens from peripheral tumor cells can enter the class I pathway for presentation by host APCs to CD8 cells, a process commonly known as "cross-presentation" (1–3). Cross-presentation has been the focus of much research over the past few years. It is an extremely efficient process and because it can induce either tolerance or immunity to Ags expressed in normal tissues, it is thought to have a role in the maintenance of self tolerance as well as the rapid clearance of viruses (2) although the overall importance of cross-presentation in vivo remains uncertain (4).

It is unknown whether apoptotic or live cells are the source of cross-presented Ag in normal tissues (1). It is essential to understand the effects of apoptosis on tumor Ag cross-presentation in vivo because such knowledge is crucial for understanding how a host interacts with established tumors and, equally importantly, how the induction of apoptosis in tumor cells, e.g., by chemotherapy, alters the efficiency of cross-presentation and the response of tumor-specific T cells.

In vitro, Ags from apoptosing cells can be cross-presented to specific CD8 cells via dendritic cells (DCs)[5] and, in some studies, macrophages and B cells (5). Studies with APCs loaded with apoptotic cells in vitro then injected into mice have produced quite variable results, some demonstrating tolerance (6–8) and others priming of CD8 responses (9–15). An in vivo study of apoptosing pancreatic islet cells demonstrated the development of tolerance after cross-presentation of islet cell Ags (16), but no clear picture is available of what is likely to occur in vivo when tumor cells apoptose. However, what happens in vivo is important, because the outcome of cross-presentation of Ags derived from tumor cells that apoptose in vivo is likely to be critical for developing effective tumor immunotherapy approaches (17, 18). Therefore, it is crucial to establish methods of evaluating the in vivo host response to tumor Ags that are cross-presented from cells made apoptotic in vivo, and whether such a process induces tolerance or activation of responding CD8 cells.

Most of the data describing the relationship between cellular apoptosis and T cell responses to Ags from such cells has been generated in part or in whole by cross-presentation experiments using in vitro systems, mostly undertaken to develop effective immunotherapy protocols (19). Although these experiments have provided important insights into APC phagocytic processes, APC subtypes, DC maturation pathways and the fate of ingested Ags, they use artificially grown APCs in a non-lymph node (LN) environment. Such experiments are essential for the development of adoptive DC immunotherapy protocols but they cannot be certain to predict the fate of in vivo apoptosing cells and their effect on

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[2] Current address: Division of Immunology, University of Connecticut Health Center, Farmington, CT 06030.

[3] Current address: Institute of Reproduction and Development, Monash University, Clayton, Australia 3168.

[4] Address correspondence and reprint requests to Dr. Bruce W. S. Robinson, Department of Medicine, University of Western Australia, Queen Elizabeth II Medical Center, 4th Floor, G Block, Nedlands, Perth, Western Australia 6009. E-mail address: bwsrobin@cyllene.uwa.edu.au

[5] Abbreviations used in this paper: DC, dendritic cell; LN, lymph node; HA, hemagglutinin; CL4, clone 4; DLN, draining LN; MLN, mesenteric LN; HNT, ●●●.

0022-1767/03/$02.00

host immune responses. This may explain why in vitro studies have produced variable results with regard to the capacity of apoptotic cells to be cross-presented by different host APCs.

To determine whether Ags cross-presented from tumor cells which apoptose in vivo are sequestered from the host Ag presentation pathways and/or induce Ag-specific tolerance, we established a model using hemagglutinin (HA) Ag-transduced tumor cells, in which apoptosis could be pharmacologically induced in vivo using the false nucleotide agent, gemcitabine. This agent is ideal for studies of in vivo induction of apoptosis because it induces cell death by apoptosis, is relatively nontoxic, can be used in vivo, and its general effects on immune responses are well-described (20–22).

The data in our study demonstrate that induction of tumor cell apoptosis in vivo not only does not sequester cell-associated Ags but actually increases tumor Ag cross-presentation, leading to priming rather than tolerance of tumor-specific CD8 T cells.

Materials and Methods

Mice

BALB/c ($H\text{-}2^d$) mice were obtained from the Animal Resources Center (Perth, Australia) and maintained under standard conditions in the Department of Medicine animal holding area (University of Western Australia, Perth, Australia). Two lines of anti-HA TCR transgenic mice were used. Clone 4 TCR transgenic mice (CL4; Ref. 23) express a TCR recognizing the dominant class I-restricted HA epitope. HNT TCR transgenic mice express a class II-restricted receptor that recognizes the dominant class II-restricted HA epitope (24). All mice used in these studies were between 8 and 12 wk of age.

Cell lines

All cell lines were regularly tested and remained negative for *Mycoplasma spp.* The murine AB1 tumor cell line is a class I^-, class II^- tumorigenic malignant mesothelioma cell line (25). Cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 20 mM HEPES, 0.05 mM 2-ME, 100 U/ml penicillin (CSL, Melbourne, Australia), 50 μg/ml gentamicin (David Bull Labs, Victoria, Australia) and 5% FCS (Invitrogen). AB1 cells were transfected with the murine influenza HA gene as previously described (AB1-HA; Ref. 26). Expression of HA was measured by FACS analysis before use in each experiment. Gemcitabine-resistant AB1-HA (AB1-HA-GR 250) was generated by culturing cells in media containing progressively increasing concentrations of gemcitabine (Eli Lilly, Indianapolis, IN). At each concentration, the cell line was passaged until growth rates were the same as the untreated parent cell line before increasing the concentration of gemcitabine. This cell line was grown and passaged in a final concentration of 1.67 μg/ml gemcitabine. The IC_{50} as assessed by the colorimetric MTT assay was >800-fold that of the parent cell line. AB1-HA-GR 250 maintained equivalent expression of the HA Ag.

Experimental protocol

AB1-HA or AB1-HA-GR250 tumor cells (1×10^6) were inoculated s.c. on one side of the ventral surface in the lower flank region. Treatment commenced 9 days later when a small palpable tumor was evident, ranging from 1–2 mm in diameter. Mice were then injected i.p. with 120 μg/g gemcitabine every third day for five doses, a regimen previously established as a maximal tolerated dose for BALB/c mice (27). Control mice received PBS vehicle alone. Mice were weighed before each dose and the dose was adjusted for individual mice. Tumor size was measured with calipers three times weekly during the course of chemotherapy and subsequently until tumor size reached ~10 × 10 mm, at which point mice were culled.

Adoptive transfer of transgenic lymphocytes

Adoptive transfer of transgenic lymphocytes was used to monitor changes in tumor-specific CD8 cell numbers in tumors by tetramer staining, lymphocyte proliferation assays, and changes in levels of in vivo CTL activity. Briefly, single cell suspensions of TCR transgenic lymphocytes were prepared from BALB/c HNT and BALB/c CL4 mice. CD4 lymphocytes were added to maintain CTL activity over the experimental time course (17). LNs were donated by i.v. injection 1 day before the first treatment dose. Cells were washed three times in PBS and counted by trypan blue exclusion after the third wash. Mice were warmed with a heat lamp and then briefly restrained for i.v. injection. Cells (3×10^7) of each of HNT and CL4 lymphocytes were injected in 200 μl.

CFSE ("Lyons-Parish") assay

To follow the fate of individual T cells throughout activation and clonal expansion, the fluorescent dye CFSE (Molecular Probes, Eugene, OR) was used. Dilution of CFSE-labeled T cells was performed as originally described by Lyons and Parish (28). Single cell suspensions were prepared from LNs harvested from HNT or CL4 mice and labeled with CFSE. Experimental groups were injected i.v. with 200 μl per mouse. Sixty-six hours after transfer, experimental mice were culled and single cell suspensions were prepared from the draining LNs (DLN), contralateral LNs, mesenteric lymph nodes (MLN), and spleens. Analysis was performed on a FACScan using CellQuest software (BD Immunocytometry Systems, San Jose, CA). For analysis of CFSE-labeled cells, 100,000 events were collected and analyzed.

Tetramer staining

Tetramers were made as follows: HA-peptide/K^d complexes were purified by HPLC size exclusion chromatography and biotinylated. Tumor-infiltrating lymphocytes were extracted by mechanical disaggregation of tumor. For analysis, 1×10^6 lymphocytes were blocked in purified anti-mouse CD16/CD32 (FcγIII/IIR; BD PharMingen, San Diego, CA), then stained with the HA tetramer for 2 h at room temperature. Samples were then incubated with FITC-labeled anti-CD8 Abs for 30 min. Data were acquired on a FACScan flow cytometer, and analyzed using CellQuest software (BD Immunocytometry Systems).

Bone marrow chimeras

BALB/c mice were lethally irradiated with 900 rad and then injected i.v. with 5×10^6 BALB/c or BALB/c $H\text{-}2^b$ T-depleted bone marrow cells (1, 2). The next day each recipient was injected i.p. with 50 μl of Thy-1-specific ascites (T24) to remove residual T cells. Mice were left to reconstitute for at least 6 wk before use.

In vivo cytotoxic T-lymphocyte assay

The "in vivo CTL assay" examines effector CTL function so it has some significant advantages over the in vitro CTL precursor expansion assay, including the avoidance of many of the in vitro artifacts generated by the latter assay and the selective expansion of CD8 populations. It was performed as previously described (17). Experimental groups were injected i.v. with 200 μl of differentially CFSE-labeled splenocytes per mouse. Eighteen hours later, experimental mice were culled and single cell suspensions were prepared from the DLN, contralateral LN, MLN, and spleens. Analysis was performed on a FACScan (BD Biosciences, Mountain View, CA) using CellQuest software. The cytotoxic ability of the host $CD8^+$ population is measured by monitoring the loss of the peptide-labeled $CFSE^{HIGH}$ peak as compared with the control $CFSE^{LOW}$ peak (as per Ref. 17).

Lymphocyte proliferation assay

Single cell suspensions of lymphocytes were washed and resuspended in RPMI 1640 with 5% FCS at 2×10^5 cells per well in 50 μl and seeded into 96-well flat-bottom tissue culture plates (BD Biosciences). Some wells were precoated with 200 μl of antimurine CD3 (mAb KT3.2; American Type Culture Collection, Manassas, VA; 1 mg/ml in PBS) overnight at 4°C, otherwise CL4 peptide was added to the cultures at concentrations of 1, 0.1, and 0.01 μg/ml. After a 48-h incubation, wells were pulsed with [^{3}H]thymidine (1 μCi/well) for 15 h, and harvested onto filter paper for scintillation counting. All assays were done in triplicate.

Statistical analysis

Data comparing differences between groups was assessed using a Student's t test or the Mann-Whitney U nonparametric test. Differences between tumor growth rates were compared using ANOVA. Kaplan-Meier survival curves were compared using the log rank test. Differences were considered significant when the p value was <0.05. Statistical analysis was conducted using the SPSS for Windows program and the GraphPad Prism program (GraphPad Software, San Diego, CA).

Results

Tumor Ag cross-presentation to host CD8 cells requires host APCs and weakly cross-primes

To confirm that the HA Ag was cross-presented by donor APCs rather than directly presented by the host tumor cells, bone marrow

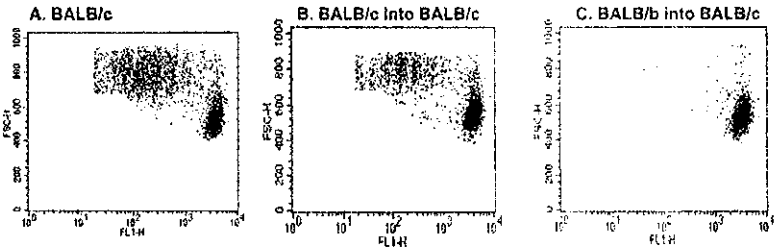

FIGURE 1. Tumor Ag cross-presentation to host CD8 cells requires host APCs. To determine whether host APCs were required for tumor Ag presentation, BALB/c mice were either studied intact (*A*) or lethally irradiated to 900 rad and then injected i.v. with 5×10^6 T-depleted bone marrow cells from BALB/c (*B*; i.e., which could present the peptide) or BALB/c.H-2^b (*C*; i.e., which could not present the peptide). Shown are CFSE proliferation analyses in the tumor-DLN of these reconstituted mice bearing the AB1-HA tumor.

reconstitution experiments were performed. HA cross-presentation, measured by adoptive transfer of CFSE-labeled CD8 cells into the animals as described in *Materials and Methods*, was restored in irradiated mice that received BALB/c marrow, i.e., with APCs that bore the H-2^d MHC restriction elements required to present the class I HA peptide to host CD8 cells. HA cross-presentation was not restored in animals that received BALB/c.H-2^b marrow, i.e., by cells that expressed H-2^b and therefore could not present this peptide (Fig. 1). Thus, only when the host bone marrow-derived APC expressed the correct MHC haplotype was HA presented to HA-specific T cells. This indicates that the tumor does not directly present HA, but requires cross-presentation by host APC.

The apoptosis-inducing agent gemcitabine shrinks the AB1-HA tumor in vivo

The in vitro sensitivity of AB1-HA to gemcitabine was established using the colorimetric MTT assay to determine the IC_{50} of the drug on this cell line. The IC_{50} of the gemcitabine-resistant cell line AB1-HA-GR250 was >800-fold higher (data not shown). To examine in vivo sensitivity to gemcitabine, mice were given standard therapy on a third daily schedule for five doses, 9 days after AB1-HA tumor inoculation. Tumor sizes ranged from just palpable to 2 × 2 mm. Mice treated with gemcitabine showed a decrease in tumor size and significant growth delay of tumors compared with control treatment ($p < 0.05$) (Fig. 2*A*). When mice bearing the gemcitabine-resistant AB1-HA-GR250 tumor were similarly treated (Fig. 2*A*), there was no change in tumor growth rate between mice receiving gemcitabine or control injections. To quantify the amount of tumor shrinkage induced by gemcitabine, the effect of gemcitabine on larger established tumors was examined by starting treatment when tumor size reached ~50 mm^2. These tumors showed an early decrease in size followed by a plateau after ~81% shrinkage on tumor volume in vivo (Fig. 2*B*). Tumor regrowth almost always occurred several days after the end of treatment.

Induction of apoptosis in vivo does not result in sequestration of tumor Ags from the cross-presentation pathway

The effect of apoptotic cell death on cross-presentation was assessed in mice inoculated with AB1-HA tumor then treated with gemcitabine, with tumor Ag-specific proliferation in vivo assessed by analyzing changes in fluorescence in adoptively transferred CFSE-labeled tumor-specific lymphocytes (Fig. 3).

In tumor-bearing mice treated with control vehicle, there was a gradual increase in tumor size and in the proportion of proliferating adoptively transferred HA-specific CD8 lymphocytes. When mice were treated with gemcitabine, the slope of the line increased significantly ($p < 0.02$), with increased proliferation seen when related to equivalent tumor size (Fig. 3*A*). For example, at a size of 40 mm^2, Ag presentation from tumor that had been treated with gemcitabine was nearly double that of animals given PBS alone (Fig. 3*B*). This demonstrates that Ag is more available for cross-presentation following gemcitabine treatment, rather than being sequestered from the cross-presentation pathway. This effect was unlikely to be due to prolonged presentation from an "earlier" tumor Ag load as Ag presentation ceased within several days when mice were "cured" of tumor and in animals that rejected allogeneic tumor (data not shown).

To determine whether this effect was due to tumor apoptosis or simply a direct effect of gemcitabine increasing the cross-presenting

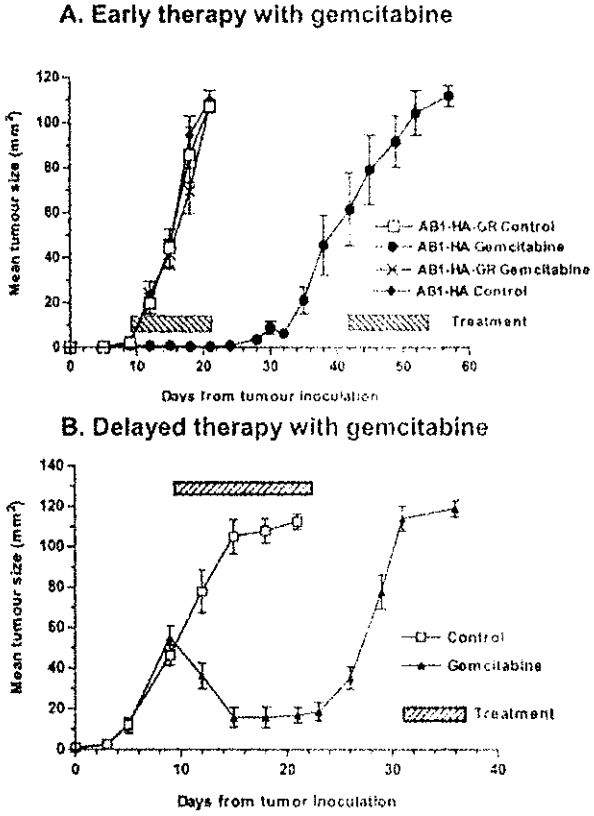

FIGURE 2. The apoptosis-inducing agent gemcitabine induces regression of the AB1-HA tumor in vivo. *A*, Early treatment. To determine whether this agent could produce tumor regression in vivo, mice (five per group) with established AB1-HA or gemcitabine-resistant AB1-HA-GR250 tumors were treated with five doses of gemcitabine or PBS injection every 3 days from days 9 to 21 after tumor inoculation, and tumor size was evaluated. *B*, Late treatment. Mice (five per group) with established AB1-HA tumors were treated with five doses of gemcitabine or PBS injection at third daily intervals from when tumors reached a mean size of 50 mm^2. Tumor size was calculated as the product of two measurements obtained using calipers. Both experiments were repeated on three occasions with similar results.

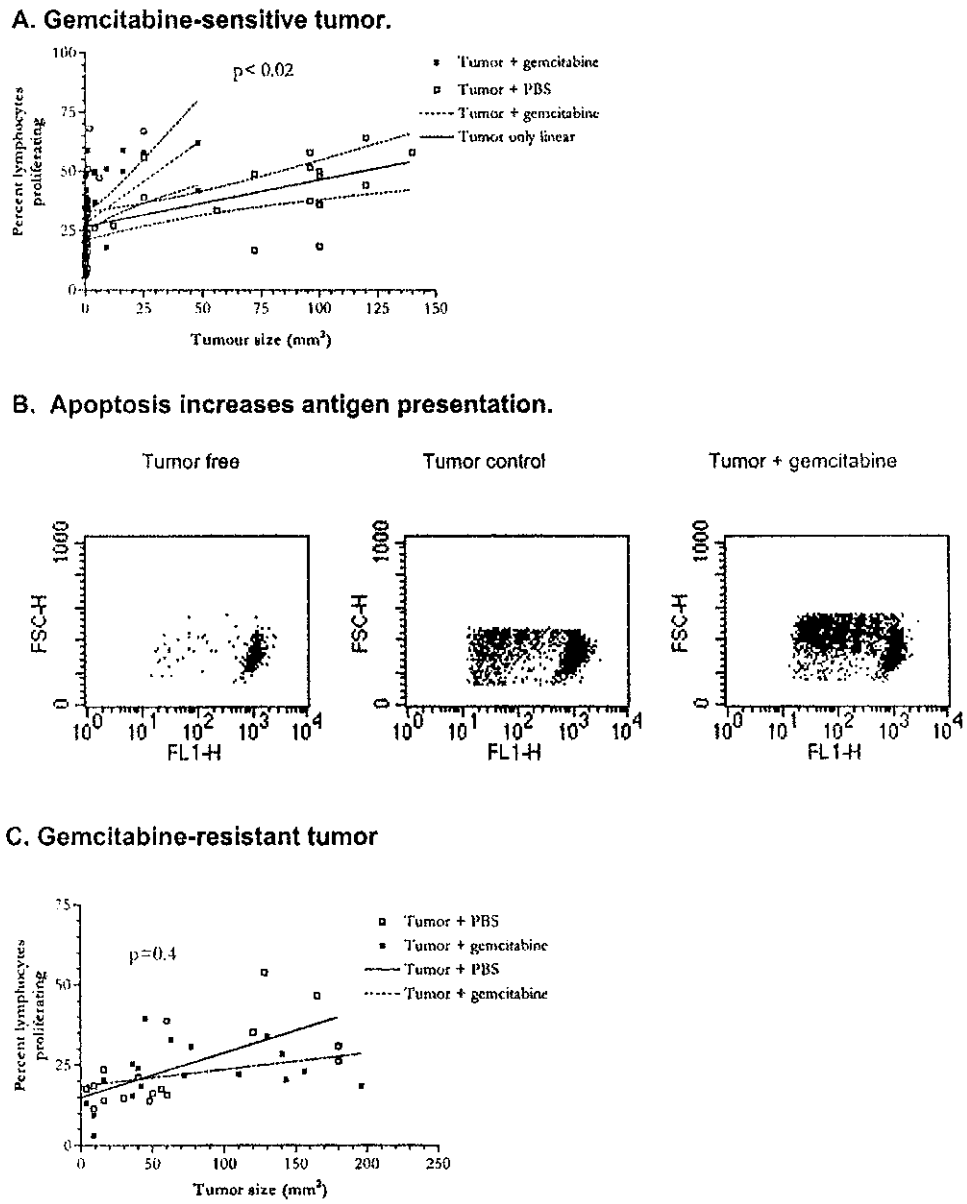

FIGURE 3. Induction of apoptosis in vivo does not result in sequestration of tumor Ags from the cross-presentation pathway. To determine whether apoptosis induced prevented removed tumor Ags from the cross-presentation pathway, mice were inoculated with (*A*) AB1-HA tumor or (*B*) gemcitabine-resistant AB1-HA-GR250 tumor, treated with gemcitabine as described above and a CFSE dilution ("Lyons-Parish") analysis was undertaken. One, 5, 13, and 21 days after gemcitabine treatment commenced, mice were adoptively transferred with CFSE-labeled cell suspension lymphocytes from naive CL4 mice. Sixty-six hours later, mice were culled and their lymphocytes were analyzed by FACS for proliferative activity, represented as a halving of fluorescence for each division. Results were plotted representing tumor size vs percent proliferating cells adjusted for division number. *C*, Representative CFSE analyses for gemcitabine-sensitive tumor at a tumor volume of 40 mm^2.

activity of APCs, this experiment was repeated using the gemcitabine-resistant cell line AB1-HA-GR250, as above. When mice bearing the gemcitabine-resistant tumor were treated, there was no significant difference in proliferative activity between animals receiving gemcitabine and those receiving control vehicle (Fig. 3*C*). Furthermore, when splenocytes of gemcitabine-treated mice were used as APCs in a lymphocyte proliferation assay requiring presentation of specific peptides to untreated HNT or CL4 lymphocytes, there was no difference in proliferation between gemcitabine-treated or control APCs (data not shown). Thus, APC function is not nonspecifically altered by gemcitabine.

Apoptosing tumor cells do not induce deletion of tumor-specific CD8 cells

We assessed deletion of tumor-specific CD8 cells after gemcitabine-induced apoptosis by using tetramers to evaluate tumor-specific CD8 cell numbers in vivo during and after treatment. Mice bearing AB1-HA tumors were treated with gemcitabine or control vehicle and DLN and spleens were removed, made into a single cell suspension and double-stained with anti-CD8 Ab (BD PharMingen) and class I^d MHC-HA tetramer complexes then analyzed by FACS to determine the percentage of $CD8^+$ T cells specific for

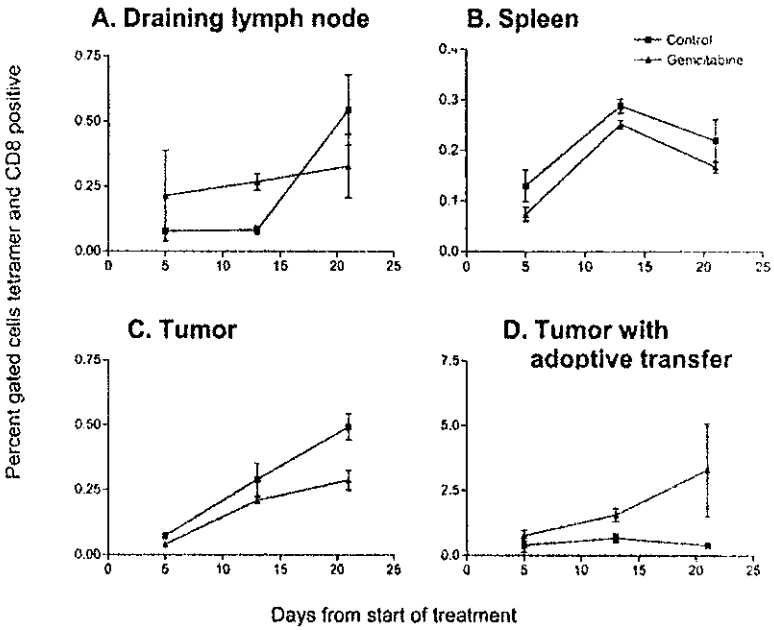

FIGURE 4. Apoptosing tumor cells do not delete CD8. To determine whether exposure of host T cells to Ags from apoptotic tumor cells induced deletion and functional tolerance, these parameters were assayed in mice inoculated with AB1-HA tumor and treated with gemcitabine or control vehicle as described. *A* and *B*, Tumor cell apoptosis does not delete tumor-specific CD8 T cells. Five to 21 days after treatment commenced, mice were culled and their DLN (*A*) and spleens (*B*) were removed and double-stained for HA-specific lymphocytes using the K^d-HA peptide-PE tetramer and CD8-FITC. The percent of lymphocytes staining double-positive for both tetramer and CD8 is shown. *C*, Tumor cell apoptosis increases tetramer$^+$ CD8 T cells in tumors. Thirteen and 21 days after treatment commenced, mice were culled and their tumors were double-stained for tumor-infiltrating lymphocytes as above. The percent of lymphocytes staining double-positive for both tetramer and CD8 is shown. *D*, Tetramer$^+$ CD8 T cells also increase in tumors following adoptive transfer of tumor-specific T cells. To increase the precursor frequency of tumor-specific T cells, 1×10^7 of each of HNT and CL4 lymphocytes were adoptively transferred 1 day before commencing treatment and tetramer analysis were performed on tumor-infiltrating lymphocytes as above.

HA. Despite exposure to increased levels of cross-presented tumor Ag, tumor-specific CD8 T cell numbers in the DLN (Fig. 4*A*) and spleens (Fig. 4*B*) of gemcitabine-treated animals were similar to those from control animals. Interestingly, when tumors were examined, there was not only no evidence of reduction of specific CD8 T cells in tumors but in fact a significant increase in the percentage of tetramer-positive cells in gemcitabine-treated animals was observed 13 days after the start of gemcitabine treatment, although there was no significant difference between the groups at 21 days (Fig. 4*C*). Because examination of a small number of lymphocytes in tumors can be inaccurate, we increased the precursor frequency of tumor-specific T cells by prior adoptive transfer and again, gemcitabine-treated animals exhibited clearly increased proportions of tumor-specific CD8 cells within tumors (Fig. 4*D*). This pattern in tumors was not present at earlier time points (data not shown). Overall there was no clear evidence that tumor-specific CD8 cells were deleted after gemcitabine-induced tumor apoptosis.

Apoptosing tumor cells do not induce functional tolerance of tumor-specific CD8 cells

Functional tolerance of tumor-specific CD8 cells after gemcitabine treatment was evaluated by assessing in vivo CTL activity in mice bearing AB1-HA tumor and treated with gemcitabine or control vehicle. CTL activity increased in both groups between days 1 and 5 and at no time was there any significant difference between gemcitabine-treated and control animals in the DLN (Fig. 5) and non-DLN or MLN. Thus gemcitabine-induced apoptosis does not lead to functional tolerance of tumor-specific CD8 cells.

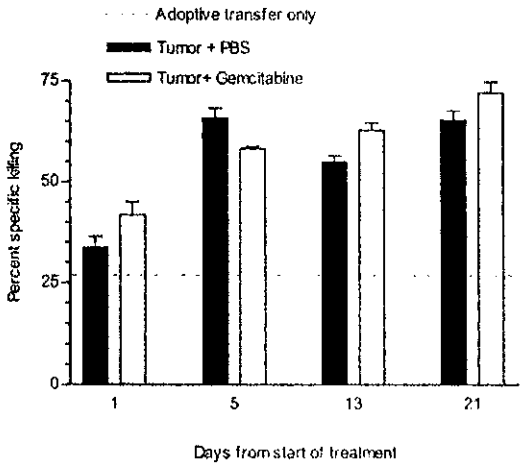

FIGURE 5. Tumor cell apoptosis does not diminish tumor-specific CD8 T cell lytic function in vivo. To determine whether apoptosis reduced the in vivo functional CTL activity of tumor specific CD8 T cells, 1–21 days after gemcitabine treatment commenced mice were injected with 2×10^7 target cells (splenocytes loaded with tumor peptide and with different CFSE concentrations ($CFSE^{HIGH}$ or $CFSE^{LOW}$). One day later, mice were culled and LN cells and spleens were examined by FACS for the ratio of $CFSE^{HIGH}$:$CFSE^{LOW}$ cells to determine the percent killing of peptide-labeled cells. Data shown are for the DLN from one experiment using five mice per experimental group. The dotted line represents the mean values seen in mice identically adoptively transferred with CL4 and HNT lymphocytes but that did not receive any tumor, and gemcitabine did not significantly alter those values. *, Statistical significance, $p < 0.05$

Apoptotic cells prime host antitumor CD8 responses in vivo

Although it was clear that the induction of tumor cell apoptosis neither deleted nor tolerized the tumor-reactive CD8 cell population, additional experiments were required to determine whether apoptotic tumor cells significantly primed the host antitumor immune response in vivo. The proliferative ability of tumor-specific $CD8^+$ T cells following gemcitabine administration was assessed as follows. Mice with small established tumors were treated with a full course of gemcitabine, culled 1, 5, 13, and 21 days after the start of gemcitabine treatment, and their lymphocytes were assessed for proliferation in response to the nonspecific stimulus anti-CD3 and to the class I HA peptide CL4. Although proliferation in response to anti-CD3 was diminished in the lymphocytes of gemcitabine-treated animals, these animals showed significantly greater proliferation to stimulation with the CL4 peptide at all time points. Day five posttreatment is shown as representative of these data (Fig. 6).

To further determine whether apoptotic cells primed host antitumor responses, we vaccinated these animals with the HA Ag-bearing PR8 influenza virus in the presence or absence of gemcitabine-induced apoptosis and evaluated tumor growth. Although a minimal delay in tumor growth was demonstrable for PR8 virus alone (Fig. 7*A*), this was consistently <10% and much smaller in magnitude and duration than the growth delay seen after gemcitabine treatment (Fig. 7*B*). Also, while there was a small increase ($p < 0.05$) in the median survival of mice receiving PR8 virus without apoptosis induction 4 (+4 days) and 8 (+7 days) days after tumor inoculation, mice treated with PR8 virus after gemcitabine treatment showed a 16-day increase in median survival ($p < 0.05$) compared with mice treated with gemcitabine alone (Fig. 7*B*). In addition, when occasional mice (< 2%) were cured of established tumor using gemcitabine alone, they were subsequently resistant to rechallenge with the same tumor and did not show any ongoing Ag presentation (data not shown). This priming effect of tumor apoptosis induction was further confirmed by studies of activating anti-CD40 Ab FGK45 administration after gemcitabine-induced apoptosis induction where synergistic effects were seen in delaying tumor regrowth and curing a proportion of mice (A. Nowak, B. W. Robinson, and R. Lake, manuscript in preparation). Thus, gemcitabine-induced tumor cell apoptosis clearly primes host antitumor responses in vivo.

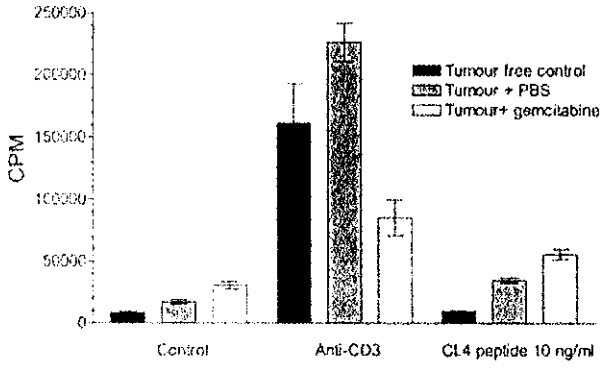

FIGURE 6. Apoptotic tumor cells prime CD8 cells. To determine whether tumor-specific CD8 T cells had been primed in vivo by tumor peptides following apoptosis induction, mice were inoculated with AB1-HA tumor and 8 days later were given HNT and CL4 transgenic lymphocytes to increase the precursor frequency of tumor-specific T cells. The following day, mice were treated with gemcitabine or PBS on a third daily schedule. Five days after starting treatment, mice were culled and their LN tested for proliferation to CL4 peptide, plate-bound anti-CD3, or no stimulus. Tumor-free BALB/c LN were also examined. Data shown represent one of three similar experiments and were obtained from four mice per group in triplicate wells. Results were similar between the three experiments. *, A value of $p < 0.05$ between gemcitabine-treated and PBS-treated groups.

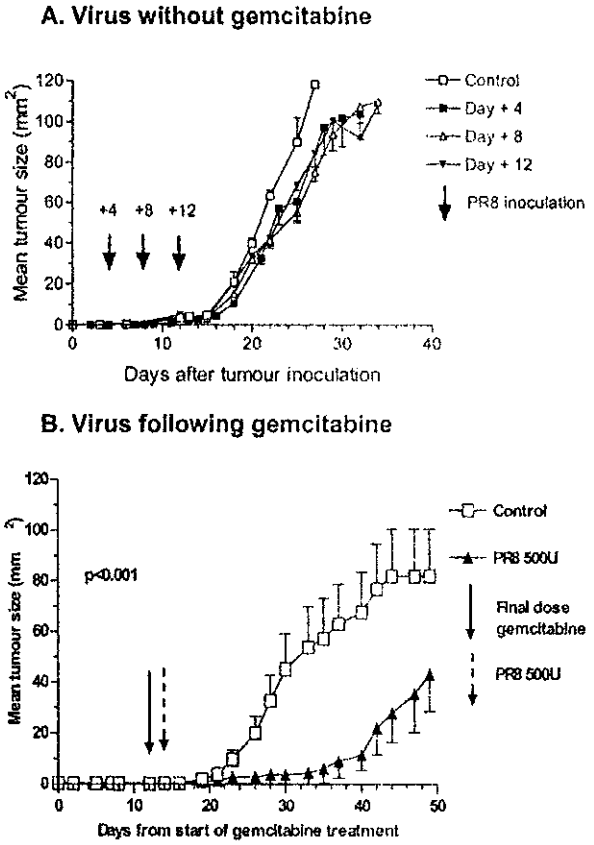

FIGURE 7. Apoptotic cells prime host antitumor immune responses in vivo. *A*, Groups of 10 mice were inoculated with AB1-HA tumor. Four, 8, and 12 days after tumor inoculation, mice were vaccinated with 500 U of PR8 virus or control vehicle i.p. without receiving any gemcitabine therapy. *B*, Groups of 10 mice were inoculated with AB1-HA tumor, treated with gemcitabine as above, then, 2 days after the final dose, were vaccinated with 500 U of PR8 virus or control vehicle i.p. Mice were monitored for tumor growth rate. Data show one of three separate experiments with similar results.

Discussion

Cross-presentation of peripheral tissue Ags has been demonstrated for a number of tissues and is thought to be important for development of tolerance and the induction of immune responses to tissue Ags (2). Although cross-presentation of tumor Ags has been demonstrated by a number of investigators, there is still uncertainty regarding the resulting response of tumor-specific CD8 cells (4). The CD8 response is likely to be influenced by the viability of the tumor cell containing the cross-presented Ag, but it remains uncertain whether the cellular source of cross-presented Ags in vivo is live or dead cells (1). Because phagocytosis of cells dying by apoptosis in vivo is exceptionally efficient, it has been theorized that tumor cell apoptosis could result in Ag sequestration (29) and that when that process is overwhelmed or fails, the host would respond by tolerance induction (8, 30). This sequence of events is integral to the "danger theory" which proposes that only necrotic cell death, particularly when associated with cellular stress, should

lead to the induction of immune responses of the sort likely to destroy tissue, e.g., antitumor responses (31). However, it has been proposed that when massive apoptosis occurs, the normally efficient phagocytic system is overwhelmed, resulting in secondary necrosis in vivo, release of proinflammatory mediators, and an increase in cross-presentation (30).

Because until now it has not been possible to accurately analyze in vivo specific tumor Ag cross-presentation and tolerance induction following in vivo apoptosis, we established a transfection-transgenic model of tumor immunity in which each of those processes could be analyzed. The transfected marker Ag enabled tumor-specific responses to be studied in vivo, and the availability of transgenic mice with large numbers of T cells expressing TCRs with specificity for that Ag enabled the level, location, and kinetics of Ag cross-presentation to be evaluated in vivo. Importantly, the tumor was sensitive to in vivo apoptosis induction by gemcitabine at doses that did not significantly otherwise alter the key immunological events being studied. The data clearly demonstrate that apoptotic tumor cells are not sequestered from cross-presentation pathways and that tumor-specific CD8 cells which respond to cross-presented tumor Ags from apoptotic cells are not deleted and are primed rather than tolerized in the process.

Tumor-derived Ag requires host APCs for presentation to host CD8 cells, i.e., is cross-presented

The requirement for host APCs demonstrated by the bone marrow chimera experiments confirms that the tumor Ag (HA) is cross-presented rather than directly presented by the tumor cells in the DLN. This is consistent with the lack of any evidence that this tumor metastasizes to LN (32). This cross-presentation of tumor Ag in DLN is efficient and tumor Ag cross-presentation is seen throughout the period of tumor growth, i.e., is constitutive (26). This parallels observations in normal rats where apoptotic bodies have been demonstrated to be constitutively present in APCs draining the normal gut, implying continuous sampling of apoptotic gut epithelial cells (33).

The only cellular source of the cross-presented Ag in our experiments was the tumor cell, although it was impossible to know whether the cells that donated tumor Ag were alive, apoptotic, or necrotic. The latter was unlikely as staining of tissue sections demonstrated no evidence of either tumor necrosis or the inflammation usually associated with necrosis in these tumors with or without gemcitabine therapy. Therefore, the source of cross-presented Ag was likely to be either apoptotic or live cells. As it is technically not possible to precisely identify which type of cell delivers tumor Ags for cross-presentation in vivo, we chose the strategy of inducing a large shift in the balance of live to apoptotic tumor cells in vivo by inducing substantial apoptosis pharmacologically, then determined how this affected cross-presentation and T cell responses.

Ag from apoptotic tissue is not sequestered from the cross-presentation pathway

As expected, in our experiments gemcitabine induced tumor cell apoptosis in vitro and caused shrinkage of tumor in vivo (20, 22). This was dependent upon the continued presence of the drug, as cessation of gemcitabine administration was associated with rapid outgrowth of the remaining tumor cells within 1 wk, mimicking clinical experience with noncurative cytotoxic chemotherapy. With the induction of massive tumor cell apoptosis, which reduced tumor volume by ~80%, no reduction in tumor Ag cross-presentation was seen. Indeed, when corrected for tumor size and hence peripheral tumor Ag load, cross-presentation of tumor Ag approximately doubled. This supports the view that apoptotic cells are efficiently cross-presented. If live cells were the only source of such Ags, cross-presentation should have declined following this large shift from live to apoptotic cells in vivo. Furthermore, our observation that the gemcitabine-treated drug-resistant line did not exhibit the same increase in tumor Ag cross-presentation confirmed that the results were due to the induction of tumor cell death and not due to any nonspecific augmenting effects of the drug itself on the cross-presenting functions of APCs.

CD8 cells specific for cross-presented Ags from apoptotic tumor cells are not deleted

Cross-presentation has been reported as a mechanism of induction of tolerance by deletion of specific $CD8^+$ T cells, a process known as "cross-tolerance," and, importantly, this has been described for the Ag used in our studies (HA). Ins-HA mice expressing the HA on the pancreatic islets do not develop insulitis. In this system, cross-presentation of islet HA occurs and is followed by rapid activation and deletion of $CD8^+$ T cells (34). Deletional tolerance has also been demonstrated following cross-presentation of self Ags from the pancreas of mice expressing the model autoantigen OVA in this site (35). Tolerization by $CD8^+$ deletion has also been demonstrated in the thymus (36). In contrast, our data do not demonstrate any decrease in numbers of HA-specific CD8 T cells following the increase in cross-presentation mediated by gemcitabine-induced apoptosis. Indeed, numbers of tumor-specific CD8 T cells within tumors increased up to 3 wk after induction of apoptosis commenced. This strongly suggests that cross-presentation of Ags from apoptotic cells is not a deletional event.

CD8 cells specific for cross-presented Ags from apoptotic tumor cells are not functionally tolerized

Sherman and coworkers (34) showed, using HA-specific T cells in the nontumor INS-HA transgenic model where HA is expressed in the pancreas, that tolerance in the CD8 compartment occurred as a consequence of cross-presentation and was dose-dependent (34). Apoptosis of pancreatic islet cells has also been demonstrated to induce tolerance in vivo (17). Therefore, it is crucial to understand the fate of CD8 cells that respond to cross-presented tumor Ags in vivo because it provides essential information for planning tumor immunotherapy studies. If apoptotic cells induce tolerance, then any increased load of apoptotic cells that ensued from administration of a cytotoxic agent like gemcitabine might be expected to provide strong tolerogenic signals, rendering immunotherapeutic strategies useless.

Our demonstration that an HA-expressing tumor induces effector CTL by day 10 and that progressive tumor growth, with its increased load of cross-presented tumor Ags, does not substantially reduce the level of CTL activity supports the view that growing solid tumors are not tolerogenic to CD8 cells per se. This is supported by our previous studies using OVA as the model tumor Ag in C57/BL6 mice where no tolerance of tumor-specific CD8 cells was observed over 4 wk of tumor growth (37). This effect contrasts to the outcome that occurs following the in vitro loading of DCs with tumor Ags, where tolerance in the CD8 compartment has been observed, a process thought to be due to a combination of maturational block, suppression of responsiveness, and the induction of suppressive molecules such as IL-10, transforming growth factor β, and PGE (7).

Overall, our data do not support a view that the induction of tumor apoptosis in vivo tolerizes the tumor-specific CD8 response.

Ags from apoptotic tumor cells cross-prime specific CD8 responses

If CD8 T cell activation was an automatic result of increased Ag cross-presentation then the induction of apoptosis in vivo by chemotherapy drugs like gemcitabine would be expected to induce some level of immunity. For example, one might expect prolonged recurrence-free intervals following chemotherapy and the detection of new delayed-type hypersensitivity skin test reactivities to tumors. This is not the usual clinical experience as most advanced cancers recur soon after cessation of chemotherapy. Clearly, in our experiments, while there is increased Ag cross-presentation and CD8 tolerance was not induced, it was important to determine whether apoptosis induction was a "null event" or in some way primed the host antitumor CD8 response. Our observation that apoptotic cell death primed CD8 T cells for an increased proliferative response to HA peptide is further supported by the demonstration that postapoptosis administration of tumor Ag-containing virus markedly slowed tumor growth. These data are consistent with the notion that the cross-presentation of tumor Ags from apoptosing tumor cells is not in fact a null event but provides a level of priming. This is confirmed by the demonstration of increased tumor-specific CD8 responses in vitro following apoptosis induction. It is also consistent with the observation that mice exhibiting complete regressions following gemcitabine therapy alone (~2% of treated animals) were immune to rechallenge (data not shown) and, indirectly, by the demonstration of increased numbers of tumor-infiltrating lymphocytes following apoptosis induction. It is possible that this priming is only apparent, and is simply a reflection of the increased Ag dose delivered to the DLN following apoptosis induction, but in other experiments increased dose alone has not been enough to prime host CD8 cells (37). It is more likely that the induction of apoptosis altered the context in which the tumor Ag was delivered. There are two main candidate mechanisms for this. First, chemotherapeutic agents can increase the level of some stress proteins expressed by cells (38), and these proteins are capable of stimulating host antitumor responses that may be sufficient to induce CD8 responses to the cross-presented tumor Ag (39). Second, the increased phagocytosis of apoptotic tumor cells by tumor-associated macrophages, one of the main sources of the proinflammatory cytokines that underlie the "cancer syndrome" of weight loss, anorexia, and lethargy, may augment the release of these proinflammatory cytokines and thus increase the responsiveness of APCs to cross-presented tumor Ags (40).

Considered together, our findings show that tumor Ags from apoptotic cells, when cross-presented to host CD8 cells, do not induce tolerance in these CD8 cells as occurs with in vitro-cultured DCs (6), nonobese diabetic mice with apoptotic pancreatic β cells (17), and class II-positive B cell tumors (41), and that a level of CD8 priming results.

Implications for tumor therapy

There have been few studies combining apoptosis-inducing chemotherapy and immunotherapy in cancer, presumably because it has been assumed that chemotherapy is so immunosuppressive that it would negate any potential benefits from immunotherapy (42).

Simultaneous administration of immunotherapy agents which prime DCs, such as IFN α and TNF-α, has been used with chemotherapy (43–46). Although this has some scientific logic, particularly in stimulating the capacity of the DCs cross-presenting the increased Ag load to promote CD8 differentiation, these combinations have often proven to be toxic. Previously published literature in this area have been limited for variety of reasons, such as an inability to study specific antitumor reactivity due to a lack of defined tumor Ags and limitations in assays available for analysis of CD8 function in vivo. One of the most widely studied agents is cyclophosphamide, which can prime CD8 responses via a process thought to include suppression of regulatory T cells (47). It is important to make it clear that gemcitabine is not simply acting in a similar way–if that were so, similar results would of course have been seen when gemcitabine was administered to gemcitabine-resistant tumors, although of course we cannot completely exclude a contributory effect of gemcitabine-induced changes in lymphocyte numbers or function in these studies.

Chemotherapy-induced apoptotic destruction of tumor cells exposes the host immune system to large amounts of tumor Ag. Although this is not enough alone to initiate a powerful antitumor response it does prime the host immune system for "adjuvant" immunotherapy. In this study it was noteworthy that ~10% of animals whose established tumors regressed following gemcitabine plus influenza vaccination showed no evidence of recurrence up to 6 mo following therapy, i.e., were "cured," a phenomenon rarely seen with the tumor used in this study or with other aggressive solid tumors. This has several implications for planning tumor immunotherapy trials. First, it suggests that the level of cross-presented tumor Ag from established tumors is not limiting. Therefore, therapy in such situations is better aimed at boosting responses to endogenous cross-presented tumor Ag rather than delivering extra Ag. Second, as drug therapy protocols using apoptosis-inducing agents like gemcitabine can prime antitumor immune responses, combining this therapy with immunotherapy is logical. Third, as postchemotherapy delivery of immunostimulation was more effective rather than pretreatment, the timing of such immunotherapy may be critical.

As not all chemotherapy induces apoptosis, not all drugs act by the same mechanisms, and individual tumors can express different mechanisms of resistance to apoptosis (48–50), we cannot conclude that all agents would have this immune priming effect. Thus this study, which provides understanding of the host T cell responses to specific tumor Ags following chemotherapy, is a foundation for deciding which immunotherapeutic approaches may enable the immune system to mount a strong antitumor response to chemotherapy-induced tumor lysis.

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Morphology:	lymphoblast 
Source:	**Disease:** lymphoma **Strain:** C57BL/6N **Cell Type:** T lymphocyte;
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	transfection host (Nucleofection technology from Lonza Roche Transfection Reagents)
Antigen Expression:	H-2b; Thy-1.2
Cytogenetic Analysis:	modal number = 39
Comments:	EL4 was established from a lymphoma induced in a C57BL mouse by 9,10-dimethyl-1,2-benzanthracene. [22448] The cells are resistant to 0.1 mM cortisol and sensitive to 20 mcg/ml PHA. A subline (EL4.IL-2, ATCC TIB-181) that produces high levels of interleukin-2 (IL-2, interleukin 2) is available. Tested and found negative for ectromelia virus (mousepox).
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: horse serum to a final concentration of 10%. **Temperature:** 37.0°C

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Medium Renewal: Every 2 to 3 days

Subculturing: Cultures can be maintained by addition or replacement of fresh medium. Start cultures at 2 X 10 exp5 cells/ml and maintain between 1 X 10 exp5 and 1 X 10 exp6 cells/ml.

Preservation: culture medium 95%; DMSO, 5%

Related Products: Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2002
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References:
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Growth Properties:	adherent (The cells form clusters in soft agar and can be adapted to suspension growth)
Organism:	*Homo sapiens* (human)
Morphology:	epithelial 
Source:	**Organ:** prostate **Tumor Stage:** grade IV **Disease:** adenocarcinoma **Derived from metastatic site:** bone
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Applications:	transfection host (Nucleofection technology from Lonza Roche Transfection Reagents)
Tumorigenic:	Yes
Antigen Expression:	HLA A1, A9
DNA Profile (STR):	Amelogenin: X CSF1PO: 11 D13S317: 11 D16S539: 11 D5S818: 13 D7S820: 8,11 THO1: 6,7 TPOX: 8,9 vWA: 17

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Cytogenetic Analysis:	The line is near-triploid with a modal number of 62 chromosomes. There are nearly 20 marker chromosomes commonly found in each cell; and normal N2, N3, N4, N5, N12, and N15 are not found. No normal Y chromosomes could be detected by Q-band analysis.
Age:	62 years adult
Gender:	male
Ethnicity:	Caucasian
Comments:	The PC-3 was initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian. [22363] The cells exhibit low acid phosphatase and testosterone-5-alpha reductase activities.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C
Subculturing:	**Protocol:** 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. 6. Incubate cultures at 37°C.
Preservation:	**Subcultivation Ratio:** A subcultivation ratio of 1:3 to 1:6 is recommended **Medium Renewal:** 2 to 3 times per week **Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2004
recommended serum:ATCC 30-2020

References:

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Research Article

Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection

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Summary The present report provides the first extensive characterization of the OT-I TCR transgenic line, which produces MHC class I-restricted, ovalbumin-specific, $CD8^+$ T cells (OT-I cells). These cells are shown to be positively selected *in vivo* in H-2^b C57BL/6 mice and in bm5 mice, which express the K^{bm5} mutant molecule. In contrast, OT-I cells were not selected by mutant K^b molecules in bm1, bm3, bm8, bm10, bm11 or bm23 mice. Interestingly, however, when positive selection was examined *in vitro* in foetal thymic organ culture (FTOC), bm1 and bm8 were still poorly selective, but the bm3 haplotype now selected as efficiently as B6. The ability to select *in vitro* correlated with the capacity to present the ovalbumin (OVA) peptide to OT-I cells, as measured by induction of an OVA-specific proliferative response. These results suggest that a lower affinity TCR:MHC interaction may be necessary for positive selection in FTOC compared with selection *in situ*.

Key words: $CD8^+$ T lymphocyte, cytotoxic T lymphocyte, ovalbumin, thymus, transgenic T cell receptor.

Introduction

The frequency of T cells specific for nominal protein antigens is extremely low. This means that it is very difficult to follow the fate of antigen-specific T cells during immune responses, although this has been achieved.[1,2] To better understand the mechanisms involved in both thymic maturation and antigenic stimulation, several laboratories have produced transgenic mice expressing a single T cell receptor specificity. We chose to generate an MHC class I-restricted TCR transgenic line specific for ovalbumin (OVA), because this is a highly manipulable antigen and there is a large body of information describing various types of immune responses to it. These OT-I mice have been briefly described previously.[3] In the present report, we further characterize the $CD8^+$ T cells produced.

Foetal thymic organ culture (FTOC) has been used to study many aspects of thymocyte development, including the role of peptides in the selection of T cells from TCR transgenic mice.[3–7] These studies support the idea that the affinity of the MHC + peptide/TCR interaction determines whether a T cell is positively selected. They also support the notion that the overall avidity of the interaction between specific peptide-bearing MHC molecules, the TCR and coreceptor molecules (CD4/CD8) regulates T cell selection. Foetal thymic organ culture has been assumed to closely mimic *in vivo* conditions for the development of T cells. In the present report, we compare the MHC requirements for selection of $CD8^+$ T cells *in vivo* with those required in FTOC. Our results indicate that MHC haplotypes incapable of selecting *in vivo* may select in FTOC. The basis of this phenomenon is examined.

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Materials and Methods

Mice

All mice, including C57BL/6 (B6), B10.BR, BALB/c, bm1, bm3, bm5, bm8, bm10, bm11 and bm23, were bred at The Walter and Eliza Hall Institute. The generation of the OVA-specific TCR transgenic line (OT-I) has been previously described.[3] These mice were originally called OVA-tcr-I, but for brevity we have changed this to OT-I. The TCR transgenes were derived from the $CD8^+$ OVA-specific T cell clone 149.42, which expresses the $V_\alpha 2$ and $V_\beta 5$ variable regions of the TCR.[8]

Flow cytometry

Analysis by flow cytometry was performed on a FACScan (Becton-Dickinson, Mountainview, CA, USA). Cells were stained with monoclonal antibodies as previously described.[9] Fluorescein isothiocyanate (FITC)-conjugated anti-CD8, phycoerythrin (PE)-conjugated anti-CD4, Streptavidin-PE and Streptavidin-TRICOLOR were purchased from Caltag Laboratories, Burlingame, CA, USA and biotin-conjugated anti-$V_\alpha 2$ was made from the B20.1 hybridoma.

Proliferative responses

Proliferative responses were performed as previously described.[10] Briefly, lymph nodes from OT-I mice were removed and single cell

suspensions prepared and washed three times. Spleen cells from B6 mice were irradiated for 1500 cGy and then coated at 5×10^7/mL with various concentrations of $OVA_{257-264}$ peptide for 1 h at 37°C. Cells were washed three times and added to wells of a 96-well flat-bottom plate at 5×10^5 cells/well in 100 µL RPMI-1640 containing 10% FCS, 2-mercaptoethanol and antibiotics (RF10). Various numbers of OT-I lymph node cells were then added to each stimulator well in 100 µL RF10 and cultured for 3 days at 37°C in 5% CO_2. The concentration of OT-I cells within the lymph node population was determined by flow cytometric staining for CD8 and $V_\alpha 2$. During the last 8 h of culture, the cells were pulsed with 37 kBq [^{3}H]-thymidine.

Foetal thymus organ culture

OT-I mice were bred onto bm1, bm3 and bm8 background animals to generate offspring with the appropriate MHC class I elements. Foetal thymic organ cultures were performed as previously described.[3,11] Briefly, foetal thymuses were explanted from 15-day-old embryos, trimmed of extraneous connective tissue and groups of 3–9 lobes maintained in culture for 7 days at 37°C in 6.5% CO_2 on nitrocellulose filters on gelfoam sponges soaked in RF10.

Cytotoxic T cell assay

Bulk cultures and the [^{51}Cr]-release assay were performed essentially as previously described.[12] Briefly, for *in vitro* restimulation of OT-I cells in bulk cultures, 10^7 OT-I spleen cells were mixed with 10^8 $OVA_{257-264}$ peptide-coated B6 spleen cells in 30 mL RF10. After 5 days, effectors were collected and concentrated in 1.5 mL for use in the [^{51}Cr]-release assay. For measurement of cytotoxicity, cultured effectors or spleen cells taken directly from OT-I mice were resuspended in 1.5 mL RF10 and then titrated in three-fold dilutions. Effectors (100 µL) were mixed with 100 µL [^{51}Cr]-labelled target cells for 4 h at 37°C and then 100 µL of supernatant was collected to determine the percentage specific lysis.

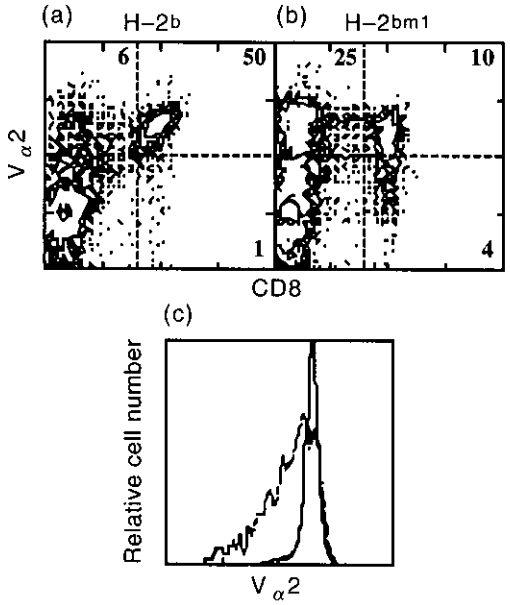

Figure 1 Expression of $V_\alpha 2$ by CD8 cells from the selecting haplotype H-2^b and the non-selecting haplotype H-2^{bm1}. Lymph node cells from OT-I mice crossed to the (a) H-2^b or (b) H-2^{bm1} haplotypes were examined by flow cytometry for expression of CD8 and $V_\alpha 2$. (c) H-2^b (——) and H-2^{bm1} (- - - -) profiles were gated on CD8$^+$ cells and $V_\alpha 2$ expression examined.

Results

Primary characterization of OVA-specific class I-restricted TCR transgenic mice

Generation of the class I-restricted OVA-specific TCR transgenic line OT-I has been briefly described previously.[3] After screening of several potential founder mice, line 243-2 was chosen because of its extensive expression of $V_\alpha 2$ (Fig. 1a,c) and $V_\beta 5$ (data not shown) by CD8$^+$ T cells. This line was termed 'OT-I' for *O*VA-specific, class *I*-restricted *T*CR transgenic. Quantification of the number of CD4$^+$ and CD8$^+$ cells indicated that transgenic mice possessed a total number of T cells similar to that of non-transgenic littermates, but that transgenic cells were strongly skewed towards the CD8$^+$ subset (Fig. 2).

To ensure that cells from the OT-I line were able to respond to OVA, lymph node cells were cultured *in vitro* with irradiated syngeneic spleen cells coated with various concentrations of the K^b-restricted $OVA_{257-264}$ peptide and proliferation was assessed on day 3 (Fig. 3). This revealed that OT-I T cells made a vigorous response to $OVA_{257-264}$ peptide. When analysed for cytotoxicity, unprimed (resting) lymphocytes from the spleen of OT-I mice did not kill specific target cells (Fig. 4a), whereas cells stimulated for 5 days *in vitro* with antigen were cytotoxic (Fig. 4b).

The expression of CD4, CD8 and $V_\alpha 2$ by OT-I thymocytes is shown in Fig. 5a,c. Consistent with the peripheral skewing of T cells towards the CD8$^+$ population, a large proportion (13%) of OT-I thymocytes were CD4$^-$8$^+$. OT-I thymi also

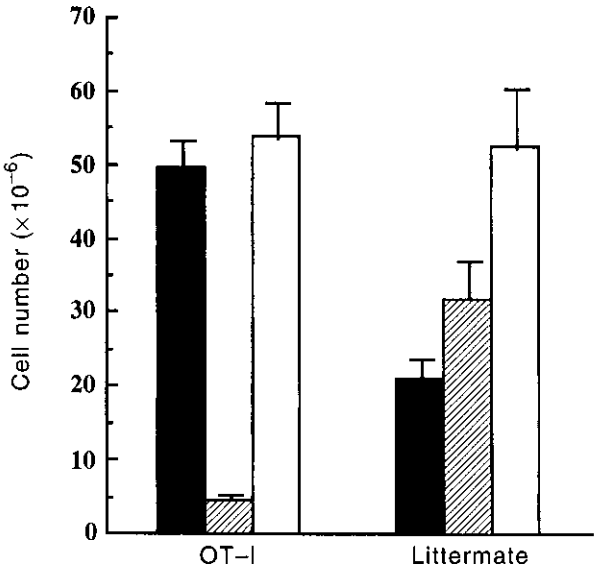

Figure 2 T cell enumeration in OT-I transgenic mice. Lymph node cells and spleen cells from 9-week-old transgenic mice and littermate controls were counted and then analysed for CD4 and CD8 expression by flow cytometry. This allowed enumeration of CD8$^+$ T cells (■), CD4$^+$ T cells (▨) and total T cells (□).

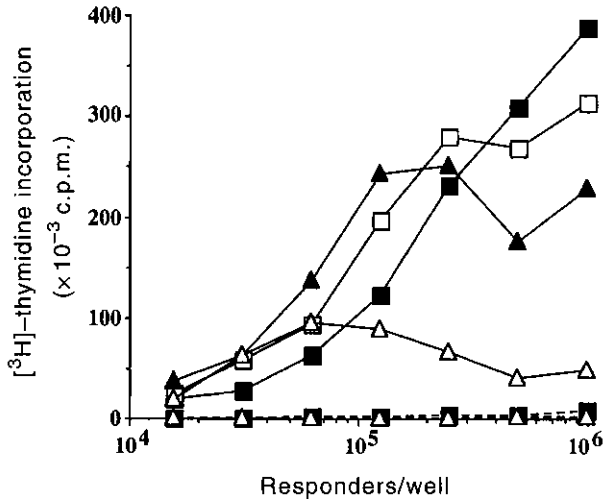

Figure 3 Proliferative response of OT-I lymph node cells to syngeneic spleen cells coated with various concentrations of ovalbumin $(OVA)_{257-264}$ peptide. Irradiated B6 spleen cells (5×10^7/mL) were coated with 0 (–△–), 0.5 (– ■ –), 5 (–△–), 50 (-▲-), 500 (–□–) or 5000 (–■–) ng/mL $OVA_{257-264}$ peptide for 1 h at 37°C. After extensive washing, these cells were cultured with various numbers of OT-I lymph node cells for 3 days and proliferation assessed by [^{3}H]-thymidine incorporation.

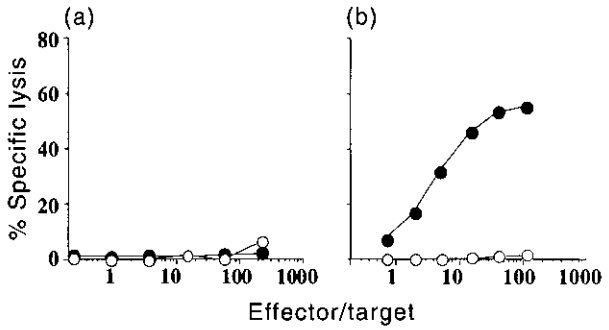

Figure 4 Cytotoxicity of OT-I cells before and after *in vitro* activation. Spleen cells from OT-I mice were measured for cytotoxicity either (a) when directly taken from mice or (b) after 5 days restimulation *in vitro*. Cytotoxicty was measured against EL4 targets alone (O) or after coating with ovalbumin $(OVA)_{257-264}$ peptide (●).

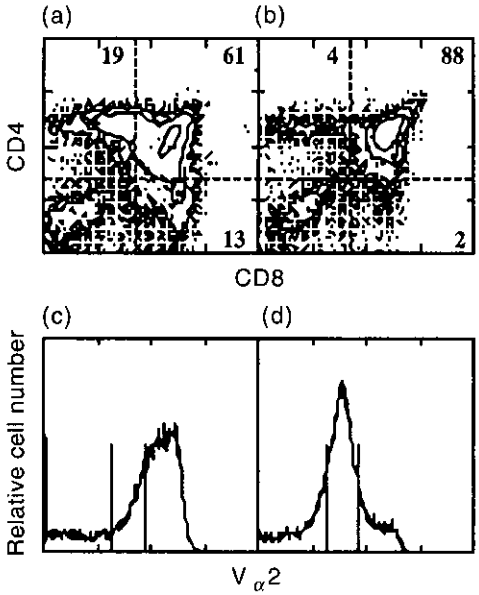

Figure 5 Expression of CD4, CD8 and $V_\alpha 2$ by OT-I thymocytes from H-2^b (B6) and H-2^{bm1} (bm1) mice. (a,c) B6 thymocytes and (b,d) bm1 thymocytes were analysed for expression of (a,b) CD4 versus CD8 and (c,d) $V_\alpha 2$.

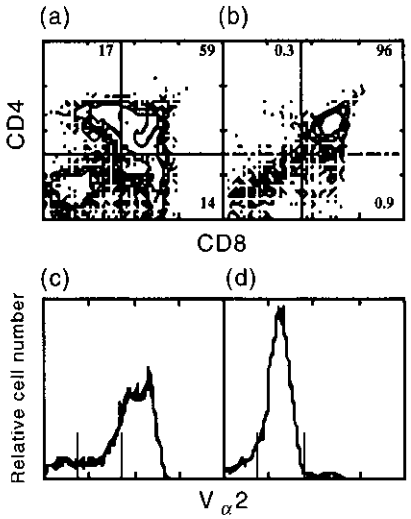

Figure 6 Expression of CD4, CD8 and $V_\alpha 2$ by RAG-1-deficient OT-I thymocytes from H-2^b (B6) and H-2^{bm1} (bm1) mice. (a,c) B6 thymocytes and (b,d) bm1 thymocytes were analysed for expression of (a,b) CD4 versus CD8 and (c,d) $V_\alpha 2$.

contained a significant proportion of cells that were $CD4^{high}CD8^{intermediate}$. These are primarily precursors of mature $CD8^+$ cells, because intrathymic transfer of such cells into normal B6 mice produced predominantly $CD4^-8^+$ cells.[13] Of note, OT-I thymocyte counts were 33 ± 8% of aged-matched, non-transgenic thymi.

To examine whether endogenous rearrangement of α chains might be responsible for some part of the thymic profile, OT-I mice were crossed onto a recombination activating gene (RAG)-1-deficient background and their thymocyte profiles compared to RAG-1-sufficient mice (compare Fig. 6a with Fig. 5a). Lack of RAG-1 expression barely altered the thymic profile, although there was a slight reduction in the proportion of cells expressing CD4 and the lowest level of CD8. These probably represent those few cells that rearranged endogenous α chains and were then selected on class II.

The effect of RAG-1 deficiency on the peripheral lymphocyte pool in OT-I mice is shown in Fig. 7. On a selecting

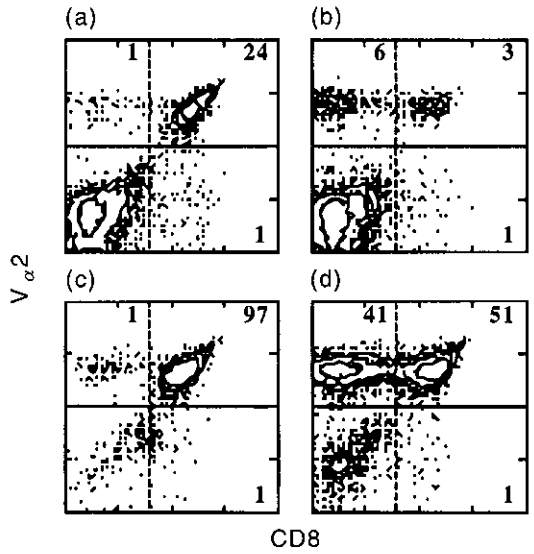

Figure 7 Expression of CD8 and $V_\alpha 2$ by RAG-1-deficient cells from the selecting haplotype H-2^b and the non-selecting haplotype H-2^{bm1}. (a,b) Spleen and (c,d) lymph node cells from RAG-1-deficient OT-I mice crossed to the (a,c) H-2^b or (b,d) H-2^{bm1} haplotypes were examined by flow cytometry for expression of CD8 and $V_\alpha 2$.

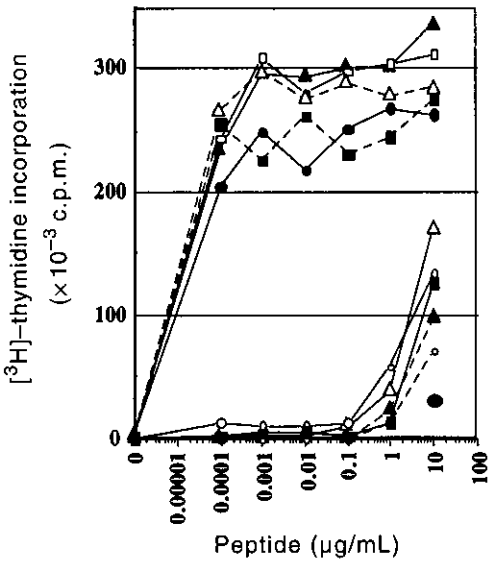

Figure 8 Proliferative response of OT-I cells to ovalbumin $(OVA)_{257-264}$ peptide presented by different MHC haplotypes. Spleen cells from various strains of mice were pulsed with OVA peptide at different concentrations and then mixed with OT-I cells of B6 origin. [^{3}H]-thymidine incorporation was examined on day 3. (–■–), B6; (–■–), bm1; (–▲–), bm3; (–□–), bm5; (–△–), bm8; (–○–), bm10; (–△–), bm11; (–●–) bm23; (–▲–), BALB/c; (–○–), B10.BR; (●), no stimulators.

haplotype, 97% of lymph node cells were now $CD8^+V_\alpha 2^+$ OVA-specific cells. In the spleen, 24% of cells expressed this phenotype. Interestingly, there was still a small population of $CD8^-V_\alpha 2^+$ cells and 50% of these cells expressed CD4 (data not shown). One unusual finding was that the total number of T cells present in the peripheral immune system of RAG-1-deficient OT-I mice was only about half that found in RAG-1-sufficient mice (data not shown). This may relate to a lack of memory cells, which have been proposed to represent about half of the T cell pool.[14]

Response to presentation of ovalbumin by H-2K^b mutant strains

To determine the antigen specificity of the TCR of OT-I mice, the capacity of the $CD8^+$ OT-I cells to proliferate in response to various H-2K^b mutant strains of stimulator cells presenting the $OVA_{257-264}$ peptide was examined. These mutants possess defined mutations in the K^b molecule, which allow definition of those residues contributing to antigen presentation.[15] H-2^k (B10.BR) and H-2^d (BALB/c) strains were also examined for presenting capacity. To prevent the cells from the responder population presenting this peptide, stimulator cells were coated with peptide and then washed. Figure 8 shows that K^b (from B6), K^{bm3}, K^{bm5}, K^{bm11} and K^{bm23} were all capable of presenting the OVA peptide, whereas K^{bm1}, K^{bm8}, K^{bm10}, H-2^d (from BALB/c) and H-2^k (from B10.BR) were not.

Positive selection

The transgenic TCR expressed by OT-I mice is K^b-restricted and has been shown to be selected by K^b.[2] We have reported that thymic skewing towards the $CD8^+$ population was not evident in the thymus of bm1 mice, which express the mutant K^b molecule K^{bm1}. We have now examined a large panel of the K^b mutants and other mice (Table 1). In these experiments, H-2^b OT-I mice were first crossed to the strain in question. If $CD8^+$ cells expressing high levels of the transgenic TCR $V_\alpha 2$ entered the peripheral lymphoid system as indicated by flow cytometric analysis, the haplotype was considered not to negatively select this TCR. To examine positive selection, F_1 mice were then recrossed to the same mutant K^b parent strain and mice homozygous for the mutant K^b background were examined for the production of cells with a high density of the transgenic TCR. Non-selecting haplotypes produced similar proportions of $CD4^+$ and $CD8^+$ cells and the $CD8^+$ cells expressed a low level of the transgenic α chain. An example of this is shown in Fig. 1b,c, where bm1 was used to examine selection. The generation of $CD8^-V_\alpha 2^+$ cells and the low level of $V_\alpha 2$ expression on the $CD8^+$ cells is indicative of rearrangement of endogenous α chains that compete with the transgenic α chain for pairing with the transgenic β chain.[9,16,17] Lack of positive selection of the transgenic TCR resulted in a thymus size that was similar to that of non-transgenic littermates.

Analysis of CD4 and CD8 expression by thymocytes in non-selecting mice such as bm1 (Fig. 5b) revealed a profile similar to that of non-transgenic littermates (data not shown). These thymii contained mainly double-positive cells, but a discrete population of CD4 single positive cells was evident. To confirm that these cells were the result of endogenous rearrangement, OT-I mice crossed to a non-selecting bm1 background were further crossed to RAG-1-deficient mice, to prevent endogenous rearrangement. Thymocytes and peripheral lymph node cells from these mice were then analysed for

Table 1 *In vivo* selection of OT-I cells in different strains of mice

Mice analysed	High TCR $V_\alpha 2$ (Positive selection)	Low TCR $V_\alpha 2$ (No positive selection)
Homozygous for MHC	B6, bm5	bm1, bm3, bm8, bm10, bm11, bm23
	High TCR $V_\alpha 2$ (No negative selection)	Low TCR $V_\alpha 2$ (Negative selection)
F_1 cross with B6 ($H\text{-}2^b$)	bm1, bm3, bm5, bm8, bm10, bm11, bm23, B6	B10.BR ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$) B10.S ($H\text{-}2^s$)

Positively selecting strains, B6, bm5.
Negatively selecting strains, B10.BR, BALB/c, B10.S.
Non-selecting strains, bm1, bm3, bm8, bm10, bm11, bm23.

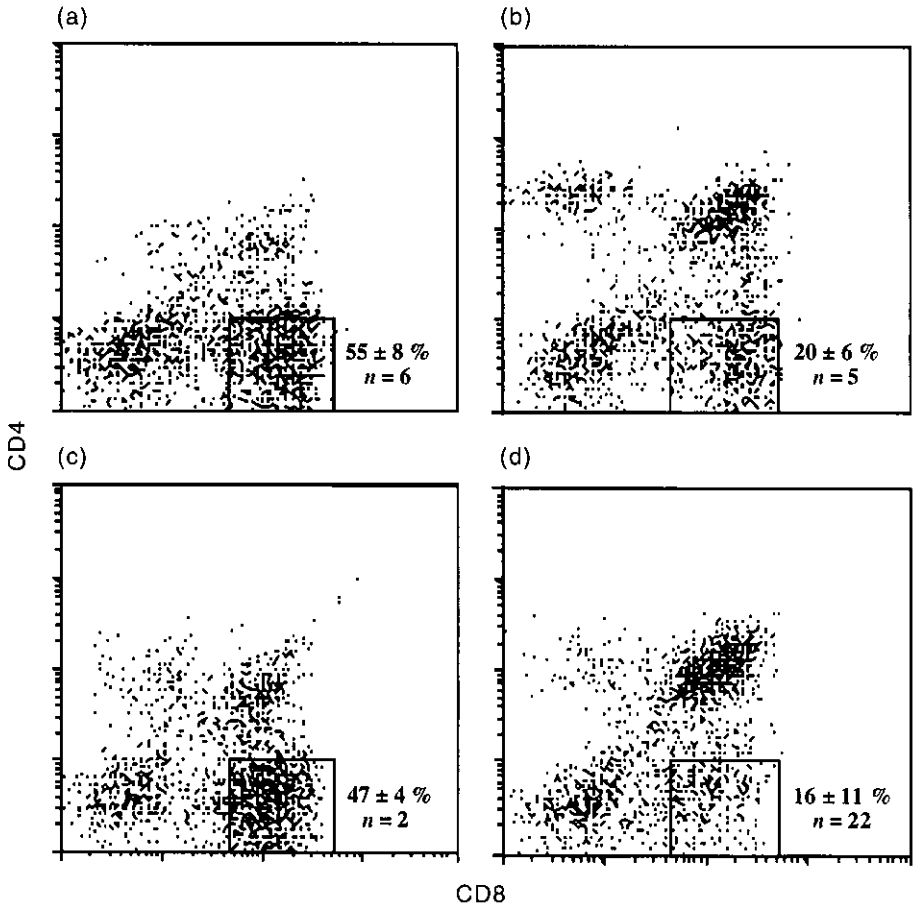

Figure 9 Selection of OT-I $CD8^+$ cells in foetal thymic organ culture (FTOC). The FTOC were generated from OT-I transgenic mice backcrossed to (a) B6, (b) bm1, (c) bm3 and (d) bm8 strains of mice. Day 15 foetal thymuses were cultured for 7 days on nitrocellulose filters. Cells isolated from these cultures were then examined by flow cytometry for expression of CD4 and CD8 and assessed for the proportion of CD8 single positive cells, as shown in the gated areas. Numbers shown are the percentage CD8 single positive cells ± the standard deviation of the mean and the number of animals examined (*n*). Panel (d) is taken from Barnden *et al.*[11]

the expression of CD4, CD8 and $V_\alpha 2$ and compared with similar mice on a selecting B6 background (Fig. 6). The RAG-1-deficient non-selecting thymi contained few CD4 single positive cells, confirming the idea that these cells were selected on endogenously rearranged TCR in RAG-1-sufficient mice. Interestingly, analysis of lymph node populations of RAG-1-deficient non-selecting bm1 mice revealed the presence of $CD8^+$ cells expressing a high density of the transgenic α chain (Fig. 7). The total number of $CD8^+$ T cells produced by bm1 mice was, however, quite small. In the experiment shown in Fig. 7, the B6 RAG-1-deficient mouse had a total of 32×10^6 $CD8^+V_\alpha 2^+$ cells, whereas the bm1 RAG-1-deficient mouse had 2.6×10^6 of these cells. Interestingly, when the $CD8^-V_\alpha 2^+$ cells from the RAG-1-deficient mice were examined for the expression of CD4, more than half of these cells on the B6 background were $CD4^+$. However, there were no $CD4^-$ cells on the bm1 background (data not shown).

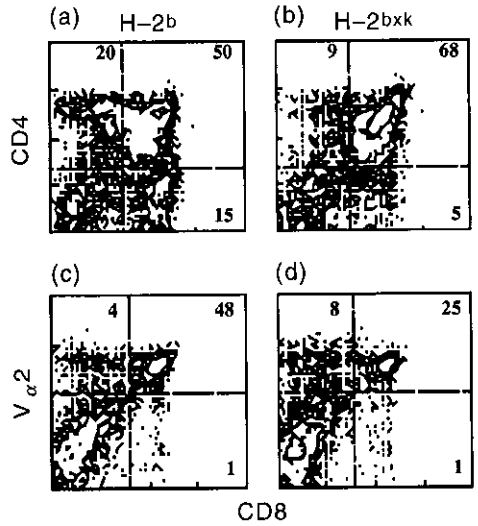

Figure 10 Negative selection of OT-I by H-2^k. The thymocytes (a,b) and lymph node cells (c,d) of H-2^b or H-2^{bxk} OT-I mice were examined for the expression of either (a,b) CD4 and CD8 or (c,d) $V_\alpha 2$ and CD8.

Positive selection of OT-I cells in foetal thymus organ culture

In studies that examined the requirements for selection of OT-I cells *in vitro*, FTOC were analysed from OT-I mice crossed to either the selecting haplotype H-2^b (B6) or non-selecting haplotypes bm1, bm3 and bm8 (Fig. 9). Surprisingly, the selection of OVA-specific CD8$^+$ cells appeared to be more efficient in FTOC than *in vivo*. The bm1 and bm8 FTOC selected relatively poorly for CD8$^+$ cells, although some CD8 single positive mature cells were generated. More importantly, bm3 now selected as efficiently as B6. One explanation for this finding was the observed up-regulation of TCR expression on double-positive cells when cultured in FTOC.[11] This may increase the overall avidity of interaction between selecting APC and thymocytes, such that the bm3 molecule is now able to provide a strong enough signal to positively select.

Negative selection

Analysis of thymic selection in different strains of mice revealed negative selection of the transgenic receptor on some haplotypes (see Table 1). Figure 10 shows the thymic and peripheral profiles of H-2^b versus H-2^{bxk} OT-I mice. The H-2^{bxk} thymi showed a reduction in the proportion of CD8 single positive cells. They were also smaller than the selecting thymus and produced fewer peripheral CD8$^+$ cells expressing the transgenic TCR. In the B10.S and BALB/c crosses, the peripheral profiles were much the same as those of the non-selecting haplotypes shown in Fig. 1 (data not shown). In contrast to the non-selecting haplotypes, however, this phenotype was generated in the F_1 generation, indicating its negatively selecting influence.

Discussion

The OT-I transgenic line is a very powerful tool for analysing both thymic development and peripheral responses of CD8$^+$ T cells.[3,5,7,11,13,18–30] The data outlined in the present report extensively characterizes this transgenic line and provides support for several conclusions that have been derived from other experimental systems. In addition, some interesting differences between CD8$^+$ T cell selection *in vivo* and in FTOC are highlighted.

Extensive skewing of the OT-I repertoire towards CD8$^+$ cells in the periphery of OT-I mice was observed (Figs 1,2) and this was reflected in their thymic profile (Fig. 5a). The requirement for TCR recognition of a class I-restricted ligand during positive selection was demonstrated by the failure of OT-I cells to be positively selected in most bm mice, which express mutant K^b molecules. In fact, other than B6 (H-2^b) mice, only bm5 mice were able to select this receptor *in vivo*. bm5 is one of the least altered mutants, with a single amino acid substitution in the floor of the antigen-binding groove, so it is not surprising that this mutant was able to select. It is interesting, however, that while bm3, bm11 and bm23 were able to present the $OVA_{257-264}$ peptide and induce proliferation of mature OT-I cells, they were unable to positively select the OT-I receptor. This suggests that the nature of class I binding to the antigenic ligand is different from class I binding to the selecting self peptide(s). The fact that the $OVA_{257-264}$ peptide can be presented by these mutant class I molecules also indicates that it is not TCR recognition of the MHC molecule surface that prevents OT-I selection. The three bm mutants, bm3, 11 and 23, each have two amino acid substitutions, one of which they have in common (position 77 D→S) and which is associated closely with the antigen-binding groove. Our findings suggest that this amino acid is important for presentation of the peptide(s) normally responsible for selecting OT-I cells. The fact that all except one of the bm mutants failed to select OT-I cells favours the idea that there are very stringent requirements for positive selection and this is likely to relate to a requirement for selection by a specific peptide or group of peptides.[20]

In studies designed to address the nature of the selecting peptide, we, in association with others,[3,20] have shown that there is specificity in the peptide required for positive selection. These studies used the FTOC system to examine selection of OT-I cells in the presence of different peptide ligands. In the present report, we examined the selection of OT-I cells by different K^b mutant molecules *in vivo* and in FTOC. We attempted to identify a K^b mutant that negatively selected the OT-I TCR in order to isolate the positively selecting peptide(s). That is, a peptide that induces negative selection when presented by a mutant K^b molecule may have lower affinity for the TCR when bound to the wild-type K^b molecule and so mediate positive selection. So far, no negatively selecting K^b mutant mice have been identified.

Because the role of peptide in the selection of CD8$^+$ cells has been examined only under conditions of low MHC expression, that is, in β2-deficient FTOC or transporter associated with antigen processing (TAP)-deficient FTOC, it has become important to confirm these findings with

more normal levels of MHC expression. For this purpose, we generated FTOC with OT-I mice crossed to the non-selecting mutant haplotypes. Our aim was to examine the types of peptides that could select in these systems. To our surprise, the level of positive selection was dramatically increased in FTOC compared with *in vivo*, particularly with the bm3 mutant, which yielded essentially the same amount of positive selection as the wild-type B6 haplotype. It is unclear why this occurs, but we speculate that this is related to the avidity of interaction generated in FTOC compared with what is apparent in a thymus *in situ*. When thymii are put into FTOC, there is an up-regulation of TCR molecules on the unselected population of $CD4^+8^+$ thymocytes.[11] This greater level of TCR expression may shift the overall avidity above the threshold for positive selection. Alternative trivial possibilities are that one of the peptides present in the FCS used in FTOC is able to cause selection of OT-I cells on the bm3 molecule or that FTOC represent early ontogenic selection that is not apparent in the older thymii. The latter is unlikely because there was little positive selection in the early neonatal period, at least in the poorly selecting bm8 situation (data not shown).

The fact that bm3 was better than bm8 and bm1 at presenting OVA peptide to OT-I cells and was also more capable of selecting OT-I cells in FTOC may be important. This could simply relate to the MHC surface seen by OT-I cells; bm3 having a better fit than bm8. Alternatively, it could relate to some link between the selecting peptide and the antigenic peptide. Although endogenous peptides unrelated to the antigenic peptide have been shown to drive OT-I positive selection,[20] it is possible that the conformations of both the antigenic peptide and the selecting peptides are somehow similar. This may be only slightly altered with bm3 compared to B6.

Finally, it is interesting that OT-I mice produced a small subset of $CD4^+$ T cells, even when on the RAG-1-deficient background. The selection of mismatched coreceptor-TCR pairs has been previously reported for the selection of a small proportion of $CD8^+$ class II-restricted haemagglutinin-specific T cells in transgenic mice that predominantly produce $CD4^+$ T cells.[31] The OT-I $CD4^+$ T cells appear to be selected at least partly by the K^b molecule, because they are not generated in bm1 RAG-1-deficient mice. As yet, we have not crossed the OT-I mice to a class II-deficient haplotype to determine whether class II is also required for selection of these $CD4^+$ T cells.

In summary, OT-I mice represent a useful transgenic line for the study of T cell development and function. The present report provides some basic characterization that will be of great value for studies in these areas.

Acknowledgements

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Cell Biology

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MFG-E8–mediated uptake of apoptotic cells by APCs links the pro- and antiinflammatory activities of GM-CSF

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) enhances protection against tumors and infections, but GM-CSF–deficient mice develop inflammatory disease. Here we show that GM-CSF is required for the expression of milk fat globule EGF 8 (MFG-E8) in antigen-presenting cells, and that MFG-E8–mediated uptake of apoptotic cells is a key determinant of GM-CSF–triggered tolerance and immunity. Upon exposure to apoptotic cells, GM-CSF–deficient antigen-presenting cells (APCs) produce an altered cytokine profile that results in decreased Tregs and increased Th1 cells, whereas concurrent ablation of IFN-γ promotes Th17 cells. In wild-type mice, MFG-E8 attenuates the vaccination activity of GM-CSF–secreting tumor cells through Treg induction, whereas a dominant-negative MFG-E8 mutant potentiates GM-CSF–stimulated tumor destruction through Treg inhibition. These findings clarify the immunoregulatory effects of apoptotic cells and suggest new therapeutic strategies to modulate CD4$^+$ T cell subsets in cancer and autoimmunity.

Introduction

The mixture of cytokines produced in the tumor microenvironment plays a decisive role in determining the outcome of the host antitumor reaction (1). Cytokines released in response to cellular stress, injury, or infection stimulate the restoration of tissue homeostasis and restrict tumor development and progression. However, persistent cytokine expression in the setting of unresolved inflammation contributes to dysregulated cell growth and apoptosis and fosters tumor cell invasion and metastasis. This dual character of host immunity in cancer pathogenesis might reflect, at least in part, the pleiotropy of cytokine activities, with specific functions dependent upon the particular array of cells and soluble factors present in the tumor microenvironment.

Substantial evidence implicates an important role for granulocyte-macrophage colony-stimulating factor (GM-CSF) in antitumor responses. Vaccination with irradiated tumor cells engineered to secrete GM-CSF evokes potent, specific, and long-lasting antitumor immunity through improved tumor antigen presentation by dendritic cells and macrophages (2). Early-stage clinical testing of this immunization strategy revealed the consistent induction of antitumor cellular and humoral responses effectuating tumor necrosis in patients with advanced solid and hematologic malignancies (3). Additional tumor destruction may be accomplished in combination with antibody blockade of CTL-associated antigen-4 (CTLA-4) (4, 5). Notwithstanding this protective activity, other work indicates that tonic GM-CSF production by tumor cells may be associated with disease progression and that high levels of the cytokine may antagonize antitumor cytotoxicity (6, 7). Thus, the overall influence of GM-CSF during tumorigenesis might vary as a consequence of distinct secretion profiles and additional factors in the microenvironment.

As tumors arise from self, the impact of GM-CSF on host reactions might also involve a contribution of this cytokine to immune regulation. GM-CSF–deficient mice develop pulmonary alveolar proteinosis (PAP), a progressive accumulation of surfactant in lung air spaces, due to impaired alveolar macrophage differentiation, which compromises surfactant clearance and catabolism (8–10). This pulmonary pathology includes an extensive lymphoid hyperplasia circumscribing pulmonary airways and veins and reflects the absence of local GM-CSF production by type II pneumocytes (11).

Aged GM-CSF–deficient mice further manifest a SLE-like disorder characterized by immune complex deposition in the renal glomeruli and autoantibodies to double-stranded DNA and C1q (12). Mice doubly deficient in GM-CSF and IL-3, a closely related cytokine that signals through the shared β_c receptor subunit, similarly display PAP and SLE (12, 13). However, concurrent ablation of IFN-γ in GM-CSF or GM-CSF/IL-3–deficient mice results in opportunistic infections and widespread inflammatory pathology, with acute and chronic granulomatous lesions present in multiple organs (12). Older compound cytokine-deficient animals succumb at high frequency to disseminated mature B cell lymphomas and diverse solid neoplasms. The prophylactic administration of antibiotics suppresses the inflammatory pathology and subsequent tumor development, establishing the interplay of microbial agents and the loss of immune homeostasis as a critical determinant of tumor susceptibility. Together, these studies reveal a key role for GM-CSF individually and in combination with IL-3 and IFN-γ in maintaining tolerance to self antigens and inhibiting tumor-promoting inflammation.

GM-CSF–deficient macrophages are impaired in the phagocytosis of apoptotic cells (12), a defect that might contribute to loss

Nonstandard abbreviations used: CCL22, CC chemokine ligand 22; CTLA-4, CTL-associated antigen-4; Flt3-L, Flt3 ligand; Gas6, growth arrest–specific gene 6; GM-CSF, granulocyte-macrophage colony-stimulating factor; MFG-E8, milk fat globule EGF 8; TRP-2, tyrosinase related protein 2.

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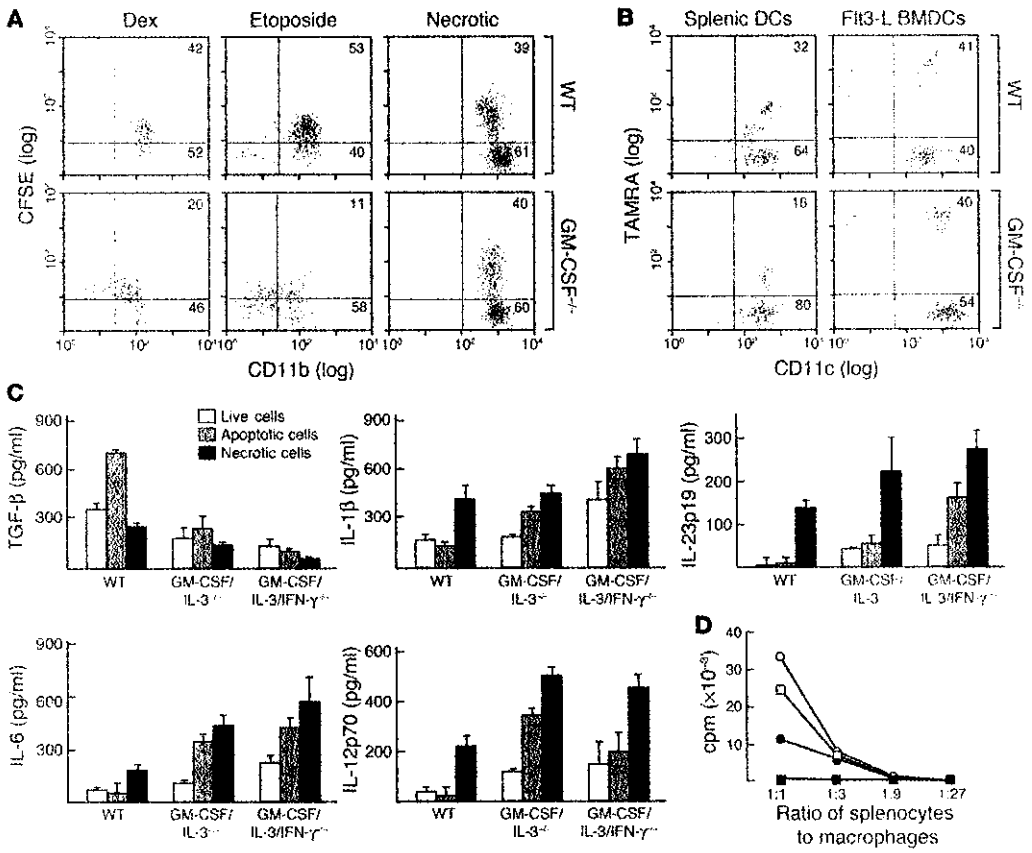

Figure 1

GM-CSF regulates the phagocytosis of apoptotic cells. (**A**) CFSE-labeled wild-type dying cells treated with dexamethasone (dex), etoposide, or necrotic cells were added to peritoneal macrophages, and phagocytosis was quantified by flow cytometry. Numbers refer to the percentage of cells within an indicated gate. (**B**) Purified splenic DCs or Flt3-L–derived bone marrow dendritic cells (BMDCs) were exposed to labeled apoptotic thymocytes, and phagocytosis was measured. (**C**) Peritoneal macrophages (3 mice per group) were loaded with apoptotic or necrotic thymocytes, and culture supernatants were measured by ELISA. (**D**) Peritoneal macrophages (circles, GM-CSF/IL-3/IFN-γ–deficient; squares, wild-type) were exposed to apoptotic (filled symbols) or necrotic (open symbols) thymocytes and cocultured with wild-type Balb/c splenocytes. Proliferation was determined by ^{3}H-thymidine uptake. Results are representative of at least 2 or 3 independent experiments.

of immune regulation, as this activity normally stimulates antiinflammatory cytokines and inhibits proinflammatory mediators (14). Antigen-presenting cells exploit multiple receptors and secreted proteins to effect the clearance of dying cells (15). Among these, oxidized phosphatidylserine exposed on the surface of apoptotic cells constitutes a major signal for phagocyte engulfment. Milk fat globule EGF 8 (MFG-E8), Del-1, and growth arrest–specific gene 6 (Gas6) are secreted opsonins that bind phosphatidylserine and promote apoptotic cell ingestion through engagement of phagocyte $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and Mer receptor tyrosine kinase, respectively (16–18). Mice deficient in these pathways develop autoimmunity and persistent inflammation (19–21), although the underlying mechanisms remain to be fully defined.

The uptake of apoptotic cells by phagocytes may influence CD4$^+$ T cell function. In allogeneic bone marrow transplantation, the infusion of apoptotic cells stimulates the generation of FoxP3-expressing Tregs through a pathway involving macrophages and TGF-β production (22). In the autologous host, the ingestion of apoptotic neutrophils by tissue macrophages suppresses tonic IL-23 secretion and thereby modulates Th17 cells as part of a homeostatic circuit regulating granulopoiesis (23). Since Treg and Th17 cell development from a common CD4$^+$ T cell precursor is influenced by TGF-β, IL-6, and other inflammatory mediators (24–26), the cytokine profile triggered by corpse clearance might contribute to CD4$^+$ T cell differentiation.

Here we delineate critical roles for GM-CSF– and MFG-E8–dependent uptake of apoptotic cells in regulating the balance of CD4$^+$ T cell subsets in tolerance and tumor protection.

Results

GM-CSF regulates the phagocytosis of apoptotic cells. GM-CSF–deficient peritoneal macrophages manifest the impaired uptake of dexamethasone-treated wild-type apoptotic thymocytes (12). Similar defects were found with etoposide-exposed Jurkat T cells and γ-irradiated splenocytes, but the uptake of freeze-thaw–induced necrotic cells was intact (Figure 1A and data not shown). GM-CSF/IL-3– and GM-CSF/IL-3/IFN-γ–deficient macrophages displayed comparable phagocytosis profiles (data not shown). CD11c-expressing splenic dendritic cells were also impaired, although Flt3 ligand–derived (Flt3-L–derived) bone marrow dendritic cells efficiently ingested

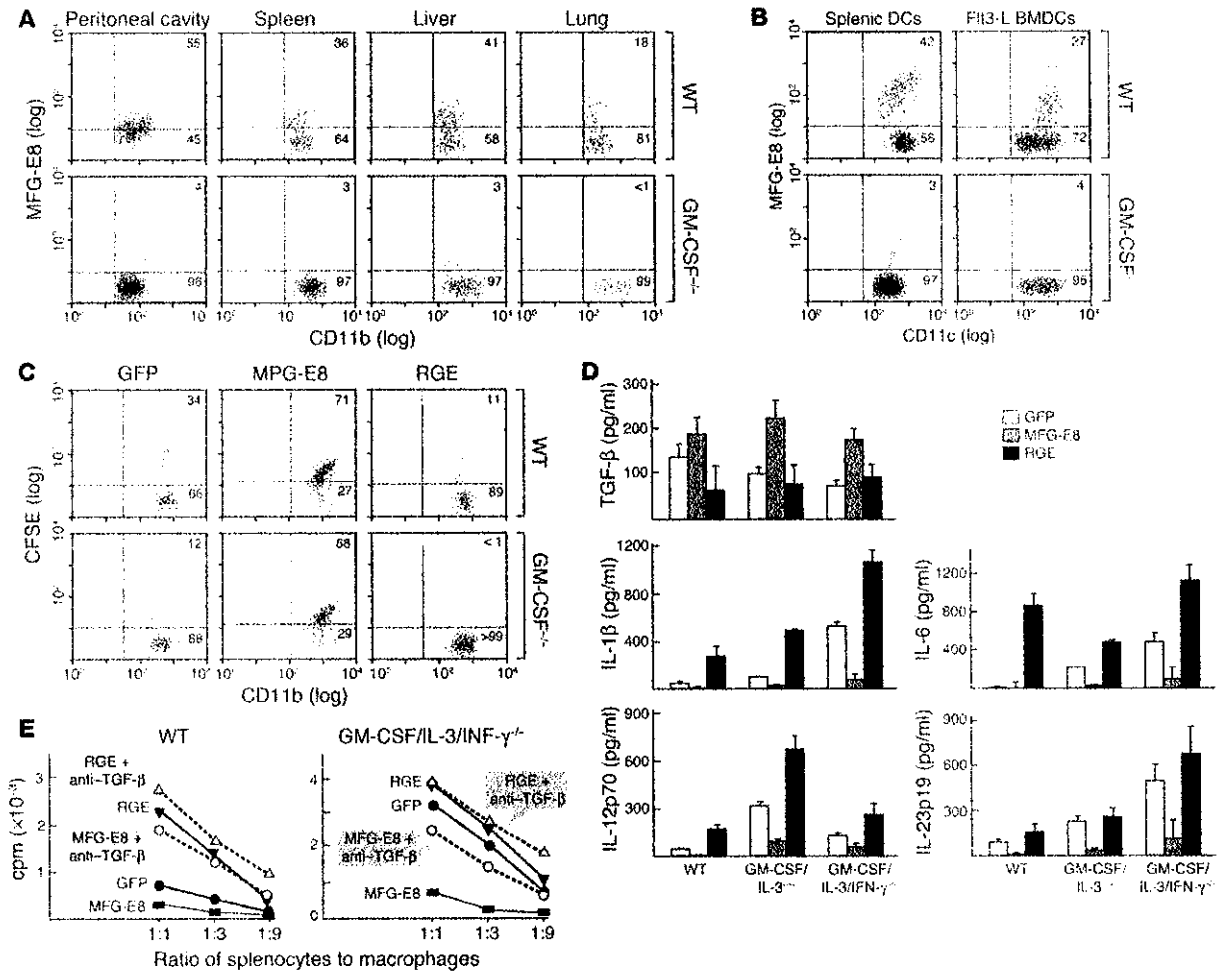

Figure 2

GM-CSF regulates MFG-E8–mediated uptake of apoptotic cells. (**A**) Purified macrophages were exposed to apoptotic cells overnight, and MFG-E8 expression was determined by flow cytometry. Numbers refer to the percentage of cells within an indicated gate. (**B**) Purified splenic dendritic cells or Flt3-L–derived bone marrow dendritic cells were exposed to apoptotic cells overnight and stained for MFG-E8. (**C**) Peritoneal macrophages were engineered to express MFG-E8, the RGE mutant, or GFP and were evaluated for the phagocytosis of labeled apoptotic thymocytes. (**D**) Transduced peritoneal macrophages (4 mice per group) were exposed to apoptotic thymocytes, and culture supernatants were analyzed by ELISA. (**E**) Transduced peritoneal macrophages were exposed to apoptotic thymocytes and then cocultured with wild-type Balb/c splenocytes with (open symbols) or without (filled symbols) neutralizing antibodies to TGF-β. Proliferation was determined by ^{3}H-thymidine uptake. Results are representative of 2 to 4 independent experiments.

apoptotic material (Figure 1B). These results are in accord with a recent report indicating that Flt3-L–generated dendritic cells acquire dying cells through a "nibbling" process, in contrast to the engulfment that is triggered by GM-CSF (27). However, our findings suggest that GM-CSF may be a more important determinant of corpse clearance than Flt3-L under steady-state conditions.

As expected, wild-type macrophages triggered substantial TGF-β but minimal IL-1β, IL-6, IL-12p70, and IL-23p19 in response to apoptotic cells (14, 23, 28), whereas necrotic cells evoked the opposite profile (Figure 1C). In contrast, GM-CSF– (data not shown), GM-CSF/IL-3–, and GM-CSF/IL-3/IFN-γ–deficient macrophages produced less TGF-β but more IL-1β, IL-6, IL-12p70, and IL-23p19 after exposure to apoptotic or necrotic cells. GM-CSF/IL-3–deficient macrophages generated the highest IL-12p70, whereas GM-CSF/IL-3/IFN-γ–deficient macrophages generated the highest IL-23p19, indicating that IFN-γ also contributes to cytokine regulation. IL-10 and TNF-α production did not differ across the set of mice (data not shown). While wild-type and GM-CSF/IL-3/IFN-γ–deficient macrophages loaded with necrotic cells stimulated robust allogeneic T cell responses, the ingestion of apoptotic cells by wild-type phagocytes suppressed proliferative responses more efficiently than GM-CSF/IL-3/IFN-γ–deficient phagocytes (Figure 1D).

GM-CSF regulates MFG-E8 expression. Previous expression profiling experiments revealed that several gene products involved in the phagocytosis of apoptotic cells were induced in microglia with GM-CSF but not M-CSF (29). Real-time PCR analysis of GM-CSF–deficient peritoneal macrophages disclosed minimal

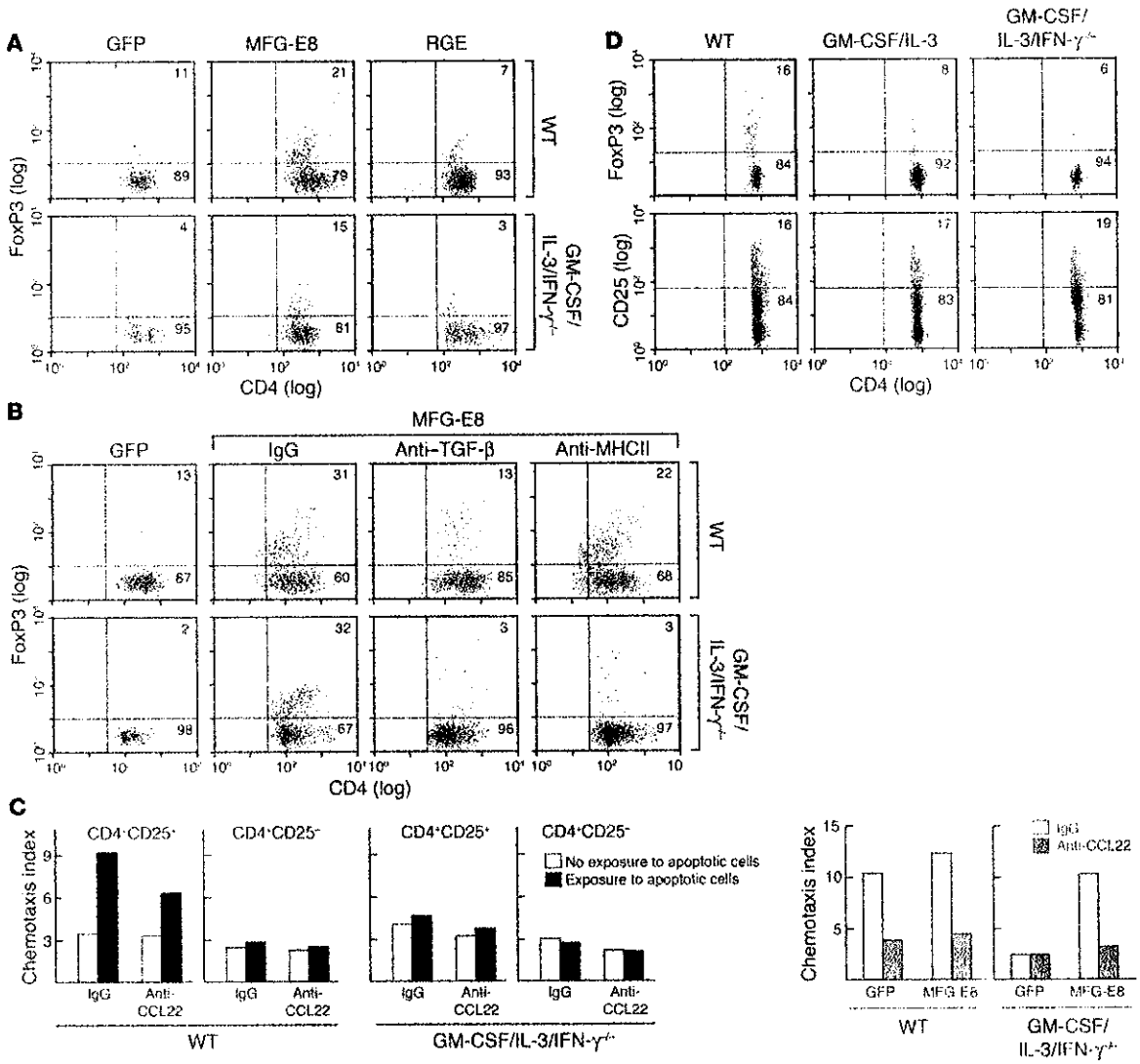

Figure 4

GM-CSF contributes to Treg homeostasis through MFG-E8. (**A**) Engineered peritoneal macrophages were exposed to apoptotic thymocytes and cocultured with wild-type syngeneic splenocytes. FoxP3-expressing Tregs were assayed by flow cytometry. Numbers refer to the percentage of cells within an indicated gate. (**B**) Blocking antibodies to TGF-β or MHC class II were added to the coculture of apoptotic cell–loaded macrophages and syngeneic splenocytes. (**C**) Supernatants from macrophages exposed to apoptotic cells were assayed for chemotactic activity against $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Blocking antibodies against CCL22 or control isotype were added as indicated. (**D**) Splenocytes were analyzed for CD4, CD25, and FoxP3 expression. Results are representative of 2 to 6 experiments.

gesting that peripheral maintenance rather than production might be the primary defect. GM-CSF/IL-3/IFN-γ–deficient $CD4^+CD25^+$ T cells showed weaker suppressor activity compared with wild-type controls (Supplemental Figure 5), although additional studies using purified $FoxP3^+$ cells are required to evaluate regulatory function on an individual cell basis.

GM-CSF regulates $CD4^+$ effector T cell subsets through MFG-E8. The increased production of inflammatory cytokines by GM-CSF–deficient antigen-presenting cells suggested that $CD4^+$ effector T cell subsets might be altered. We thus cocultured syngeneic wild-type $CD3^+CD45RA^+CD62^{hi}$ naive T cells with apoptotic cell–loaded peritoneal macrophages and analyzed IL-17 and IFN-γ expression by flow cytometry (Figure 5A). Consistent with the augmented IL-12p70 secretion, GM-CSF/IL-3–deficient macrophages stimulated increased Th1 cells. The combination of augmented IL-23, IL-1β, and IL-6 levels in GM-CSF/IL-3/IFN-γ–deficient macrophages yielded increased Th17 cells. MFG-E8 transduction of cytokine-deficient macrophages suppressed $CD4^+$ effector cell responses. Comparable findings were obtained using Flt3-L–derived bone marrow dendritic cells (Figure 5B). Expression of the RGE mutant in wild-type macrophages promoted Th1 cells, whereas transduction of IFN-γ–deficient macrophages triggered Th17 cells (Figure 5C). Moreover, splenocytes stimulated with PMA and ionomycin showed Th1 skewing in

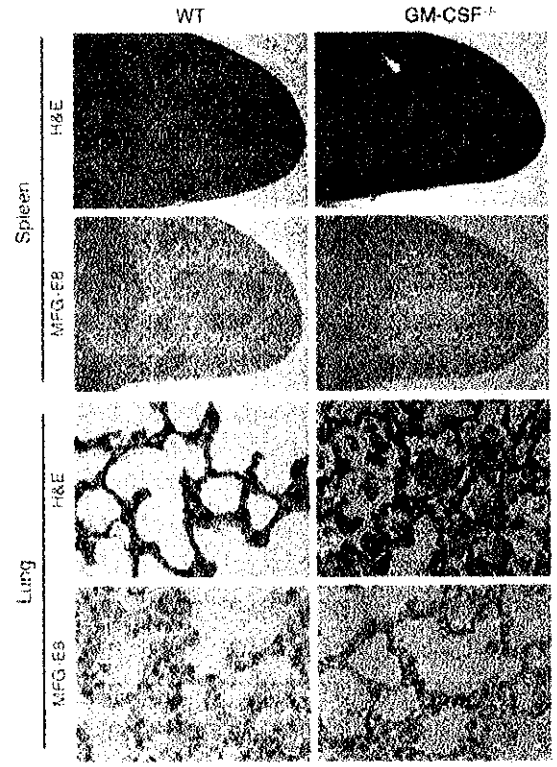

Figure 3
GM-CSF regulates steady-state MFG-E8 expression in antigen-presenting cells in vivo. Anti–MFG-E8 staining was detected in germinal center macrophages and pulmonary alveolar macrophages of wild-type but not GM-CSF–deficient mice. The eosinophilic material in the alveoli of GM-CSF–deficient mice is pulmonary surfactant. Original magnification, ×25 (spleen), ×250 (lung).

or no changes in the levels of MARCO, CD36, scavenger receptor-A, and the putative phosphatidylserine receptor, although MFG-E8 transcripts were significantly reduced compared with wild-type cells (data not shown). Flow cytometry confirmed the marked decrease in MFG-E8 expression in macrophages isolated from the peritoneal cavity, spleen, liver, and lung of GM-CSF–deficient mice (Figure 2A). While MFG-E8 was detected by immunohistochemistry in germinal center macrophages and alveolar macrophages of wild-type mice, consistent with previous reports (20), no staining was found in the spleens or lungs of GM-CSF–deficient animals (Figure 3) or in thymic macrophages from either strain (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI30966DS1). Splenic dendritic cells and Flt3-L–derived bone marrow dendritic cells from GM-CSF–deficient mice also showed reductions in MFG-E8 compared with wild-type levels (Figure 2B), while similar decreases were observed with GM-CSF/IL-3 and GM-CSF/IL-3/IFN-γ–deficient antigen-presenting cells (data not shown). Modest reductions in $\alpha_v\beta_5$, Gas6, and Mer were further detected (Supplemental Figure 2), suggesting that GM-CSF broadly regulates phosphatidylserine-based uptake of apoptotic cells.

To clarify the contribution of MFG-E8 deficiency to the impaired uptake of apoptotic cells, we used retroviral transduction to reconstitute MFG-E8 expression in peritoneal macrophages in vitro. A high-titer virus encoding a previously described MFG-E8 mutant, in which the RGD sequence involved in integrin binding was modified to RGE (30), was also generated. This protein retains the capacity to bind phosphatidylserine on apoptotic cells but cannot be internalized and thereby functions as a dominant-negative inhibitor. Flow cytometry documented that transduced cytokine-deficient macrophages achieved MFG-E8 levels comparable to those of wild-type cells (data not shown). MFG-E8 restoration increased the phagocytosis of apoptotic cells in GM-CSF–deficient cells to wild-type levels, whereas MFG-E8 over-expression in wild-type cells further augmented corpse ingestion (Figure 2C). Confocal microscopy demonstrated that MFG-E8 mediated engulfment of apoptotic cells rather than simply mediating surface binding to phagocytes (Supplemental Figure 3). MFG-E8 levels did not alter the uptake of necrotic cells (data not shown). In contrast, the RGE mutant decreased apoptotic cell ingestion in wild-type and cytokine-deficient macrophages.

Enforced MFG-E8 expression in mutant macrophages restored TGF-β and reduced IL-1β, IL-6, IL-12p70, and IL-23p19 to levels comparable to wild-type controls (Figure 2D). Conversely, the RGE mutant decreased TGF-β and increased IL-1β, IL-6, IL-12p70, and IL-23p19 in wild-type macrophages. The RGE mutant also abrogated the inhibitory effects of apoptotic cells on the allostimulatory activity of wild-type peritoneal macrophages (Figure 2E), while MFG-E8 transduction normalized the aberrant allostimulatory activity of GM-CSF/IL-3/IFN-γ–deficient macrophages exposed to apoptotic cells. This suppression required TGF-β, as the addition of anti–TGF-β–neutralizing antibodies antagonized the effects of MFG-E8 but not those of the RGE mutant.

GM-CSF is required for Treg homeostasis through MFG-E8–mediated uptake of apoptotic cells. To explore whether the phagocytosis of apoptotic cells might influence Tregs, we cocultured syngeneic wild-type $CD4^+$ T cells with apoptotic cell–loaded peritoneal macrophages and then analyzed FoxP3 expression (Figure 4A). Wild-type macrophages efficiently stimulated Tregs, but GM-CSF/IL-3/IFN-γ cells were impaired. MFG-E8 transduction of both wild-type and cytokine-deficient macrophages increased $FoxP3^+$ cells, although the RGE mutant decreased Tregs. Antibody blocking experiments established that MFG-E8–mediated Treg expansion required TGF-β and MHC class II (Figure 4B). The $FoxP3^+$ cells proliferated during the coculture and manifested typical suppressor activity in allogeneic mixed leukocyte reactions (data not shown). Moreover, supernatants from apoptotic cell–loaded wild-type but not GM-CSF/IL-3/IFN-γ–deficient macrophages provoked selective migration of $CD4^+CD25^+$ T cells in a CC chemokine ligand 22–dependent (CCL22-dependent) fashion (Figure 4C). Reconstitution of MFG-E8 expression in mutant macrophages restored chemotaxis (31).

Consistent with these results, the frequency of $CD3^+CD4^+FoxP3^+$ T cells in GM-CSF– (data not shown), GM-CSF/IL-3–, and GM-CSF/IL-3/IFN-γ–deficient mice were significantly decreased compared with wild-type controls (Figure 4D). The absolute numbers of $FoxP3^+$ cells were $3.5 \pm 1.8 \times 10^5$ (mean ± SD) cells/spleen for 5 wild-type mice, $0.7 \pm 0.1 \times 10^5$ cells/spleen for 5 GM-CSF/IL-3–deficient mice, and $0.7 \pm 0.4 \times 10^5$ cells/spleen for 5 GM-CSF/IL-3/IFN-γ–deficient mice ($P = 0.007$, both knockout strains compared with wild-type). Similar Treg reductions were observed in lymph nodes but not thymi of mutant mice (Supplemental Figure 4), sug-

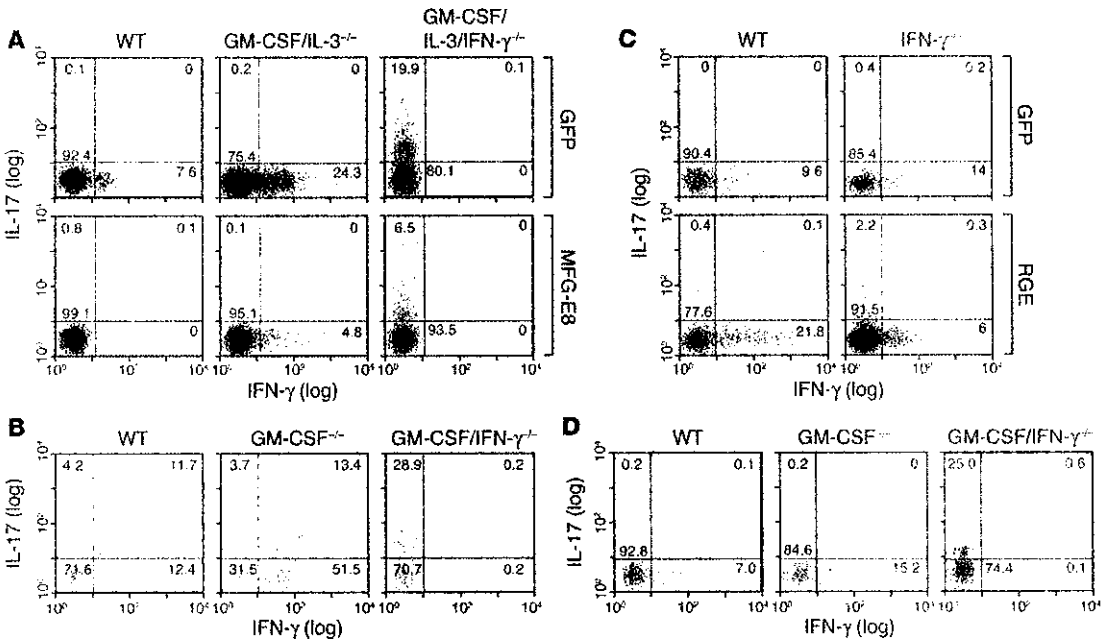

Figure 5

GM-CSF regulates CD4+ effector T cells through MFG-E8. **(A)** Peritoneal macrophages were exposed to apoptotic thymocytes and cocultured with syngeneic wild-type CD3+CD45RA+CD62^{hi} naive T cells. T cells were then analyzed for IL-17 and IFN-γ production. Numbers refer to the percentage of cells within an indicated gate. **(B)** Flt3-L–derived bone marrow dendritic cells were exposed to apoptotic thymocytes and used to stimulate syngeneic wild-type naive T cells. **(C)** Engineered macrophages were exposed to apoptotic thymocytes and used to stimulate syngeneic wild-type naive T cells. **(D)**. Splenocytes were stimulated with PMA and ionomycin, and cytokine production of gated CD3+CD4+ cells was determined by flow cytometry. Similar results were obtained in at least 2 experiments.

GM-CSF–deficient mice and Th17 skewing in GM-CSF/IFN-γ–deficient mice (Figure 5D).

MFG-E8 reconstitution normalizes CD4+ subsets in GM-CSF–deficient mice. To test further the role of MFG-E8 in GM-CSF–dependent responses in vivo, we performed bone marrow transplant experiments. Hematopoietic progenitors from wild-type and GM-CSF/IL-3/IFN-γ–deficient mice were transduced with retroviral vectors and infused into lethally irradiated 2- to 4-month-old recipients. Mice were sacrificed 2 months after transplant, and GFP/MFG-E8 expression was documented in macrophages (data not shown). MFG-E8 increased phagocytosis of apoptotic cells in wild-type and GM-CSF/IL-3/IFN-γ–deficient macrophages, whereas the RGE mutant reduced corpse clearance in both strains (Figure 6A). MFG-E8 expression increased TGF-β and reduced IL-6, IL-23p19, and IL-17 levels in the sera of wild-type and GM-CSF/IL-3/IFN-γ–deficient mice (Figure 6B). In contrast, the RGE mutant decreased TGF-β but increased IL-6, IL-23p19, IL-17, and IL-12p70 levels in the sera of both strains. The RGE mutant also evoked circulating IFN-γ in wild-type mice. This increase in proinflammatory cytokines might account for the low incidence of peri-transplant mortality observed only in animals that received RGE-expressing bone marrow (data not shown). Flow cytometry revealed that MFG-E8 increased FoxP3+ Tregs in wild-type and GM-CSF/IL-3/IFN-γ–deficient splenocytes, whereas the RGE mutant decreased Tregs in wild-type mice (Figure 6C). MFG-E8 suppressed, but the RGE mutant increased Th17 cells in GM-CSF/IL-3/IFN-γ–deficient mice, while the RGE mutant augmented Th1 cells in wild-type recipients (Figure 6D). MFG-E8 also restored the immunosuppressive effects of apoptotic cells in GM-CSF/IL-3/IFN-γ–deficient mice, as documented by the reduced allostimulatory activity (Figure 6E). Taken together, these findings establish a requirement for MFG-E8 in the immunoregulatory activities of GM-CSF.

Antigen-presenting cell maturation involves MFG-E8 downregulation. Since MFG-E8 plays a critical role in GM-CSF–triggered tolerance, GM-CSF–induced protective immunity might involve downregulation of MFG-E8 function. Indeed, previous work showed robust MFG-E8 expression in immature, GM-CSF–derived bone marrow dendritic cells, whereas LPS-evoked maturation decreased MFG-E8 (32). Here we found that wild-type peritoneal macrophages treated with diverse TLR agonists including peptidoglycan (TLR2), poly-I-C (TLR3), LPS (TLR4), or CpG oligonucleotides (TLR9) suppressed MFG-E8 induction upon exposure to apoptotic cells (Figure 7A). Enforced MFG-E8 expression antagonized the reduction in TGF-β and increase in IL-6 stimulated by LPS (Figure 7B) and suppressed the enhanced allostimulatory activity of apoptotic cell–loaded macrophages treated with LPS (Figure 7C).

B220+, but not B220−, Flt3-L–derived bone marrow dendritic cells efficiently cross-present apoptotic cell antigens to stimulate CD8+ cytotoxic T cell responses (27). MFG-E8 expression was restricted to B220+ dendritic cells, whereas maturation with LPS or necrotic cells downregulated MFG-E8 in these and GM-CSF–derived dendritic cells (Figure 7D). Sorted B220+, but not B220−, Flt3-L–derived dendritic cells secreted TGF-β upon exposure to apoptotic cells but not necrotic cells (Figure 7E). In contrast, B220+, but not B220−, dendritic cells produced IFN-α in response to apoptotic cells, and this was increased with LPS, as expected (27). B220+,

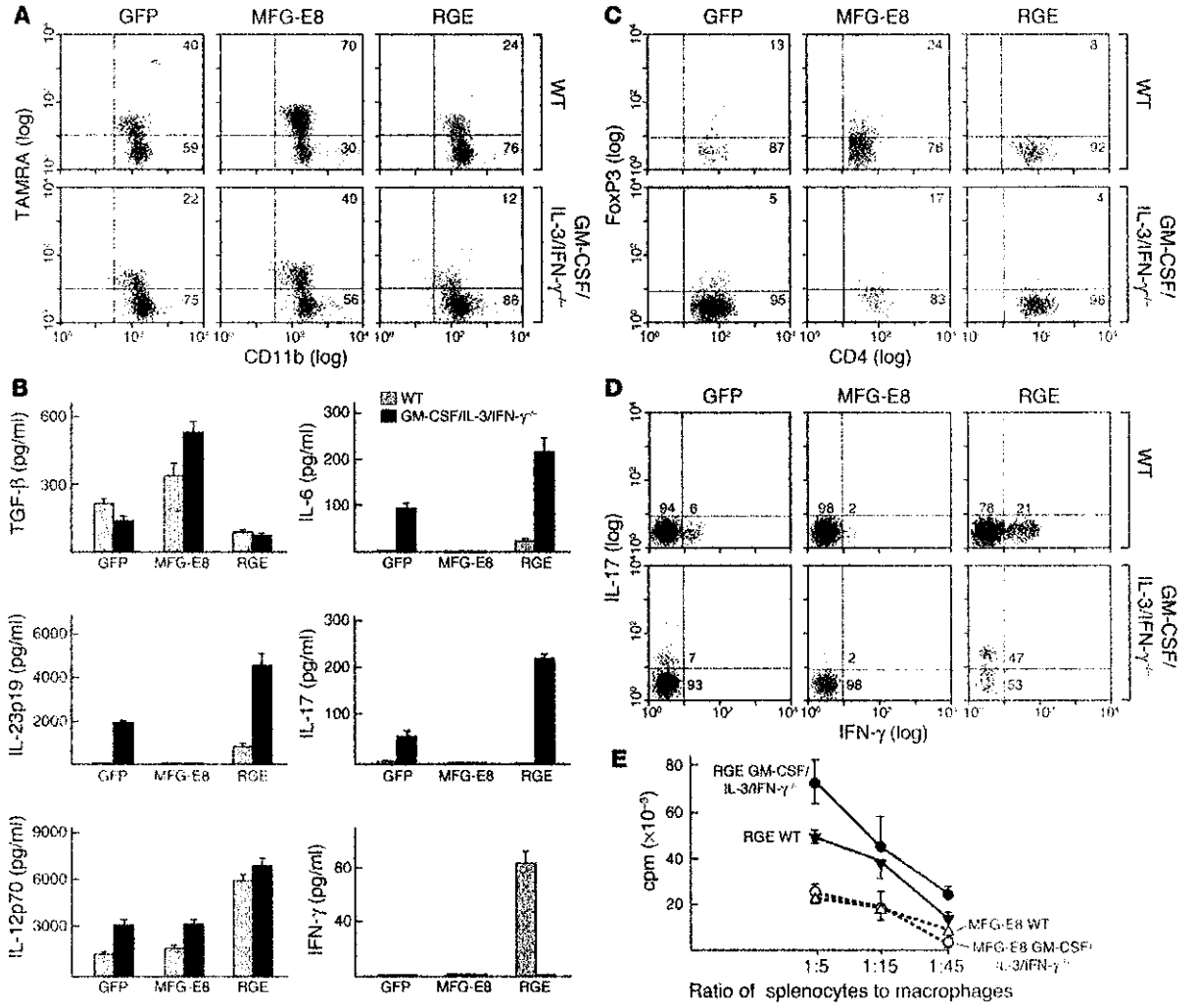

Figure 6

MFG-E8 reconstitution restores CD4+ T cell homeostasis in vivo. (**A**) Peritoneal macrophages recovered 2 months following transplantation (5 mice per group) were assayed for phagocytosis of labeled apoptotic thymocytes. Numbers refer to the percentage of cells within an indicated gate. (**B**) Serum cytokine levels measured by ELISA 2 months after transplant ($n = 4$). (**C**) Splenocytes ($n = 4$) were harvested 2 months after transplant and assayed for FoxP3 expression and (**D**) IL-17 and IFN-γ expression (CD3+CD4+ gated cells). (**E**) Peritoneal macrophages from mice that received transplants were loaded with apoptotic cells and used to stimulate allogeneic Balb/c splenocytes. Proliferation was determined by ^{3}H-thymidine uptake. Similar results were obtained with 2 independent transplant experiments.

but not B220+, dendritic cells also produced IFN-α in response to necrotic cells. These results, together with our observation that Flt3-L–derived dendritic cells can ingest apoptotic cells through an MFG-E8 independent pathway (Figure 1B), support the idea that MFG-E8 expression in particular dendritic cell subsets is linked to the induction of tolerance rather than protective immunity.

MFG-E8 regulates the antitumor effects of GM-CSF–secreting vaccines. To examine the impact of MFG-E8 on GM-CSF–stimulated protective responses, we utilized the B16 melanoma model (2). In this system, vaccination with irradiated, GM-CSF–secreting tumor cells protected wild-type syngeneic C57BL/6 mice from subsequent challenge with live wild-type B16 cells, whereas vaccination with irradiated, parental B16 cells was ineffective (Figure 8A). Immunization with B16 cells expressing the RGE mutant failed to protect against tumor challenge, indicating that blockade of phosphatidylserine was not sufficient for protective immunity in this system. Vaccines composed of B16 cells secreting MFG-E8 were similarly inactive. However, coexpression of MFG-E8 abrogated the protective immunity elicited with GM-CSF–secreting tumor cells, whereas the RGE mutant did not.

To determine whether the RGE mutant might augment the antitumor effects of GM-CSF, we utilized a therapy model in which vaccination was begun on the same day as tumor challenge (Figure 8B). Under these conditions, irradiated, GM-CSF–secreting B16 cells evoked a modest delay in tumor growth, but all animals eventually succumbed to progressive tumor. MFG-E8 coexpression also inhibited the impact of GM-CSF–secreting tumor cells in this system. Nonetheless, the RGE mutant potentiated GM-CSF–induced

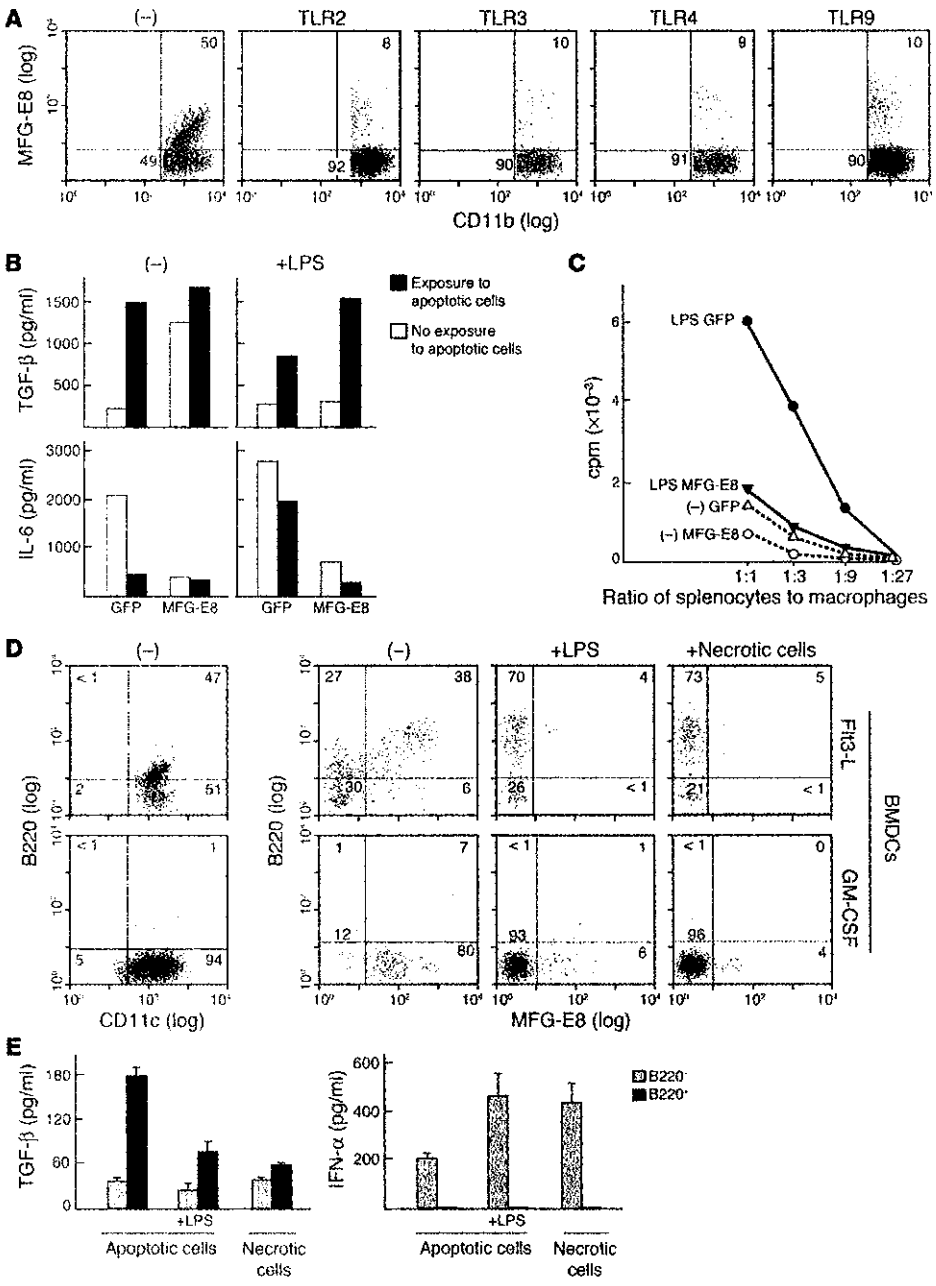

Figure 7

MFG-E8 expression is downregulated upon antigen-presenting cell maturation. (**A**) Wild-type peritoneal macrophages were exposed to apoptotic cells and peptidoglycan (TLR2), poly-IC (TLR3), LPS (TLR4), or CpG (TLR9), and MFG-E8 expression was determined. Numbers refer to the percentage of cells within an indicated gate. (**B**) Engineered wild-type peritoneal macrophages were exposed to apoptotic cells with or without LPS, and supernatants were assayed by ELISA. (**C**) Transduced peritoneal macrophages were exposed to apoptotic cells with or without LPS and cocultured with allogeneic Balb/c splenocytes. Proliferation was determined by ^{3}H-thymidine uptake. (**D**) Flt3-L– or GM-CSF–derived bone marrow dendritic cells were assayed for B220 and MFG-E8 expression. LPS or freeze-thaw–induced necrotic cells were added where indicated. (**E**) Flt3-L–derived dendritic cells were sorted into B220^{+} and B220^{-} populations, exposed to apoptotic or necrotic cells, and assayed for cytokine production by ELISA. Similar results were obtained in at least 2 independent experiments. –, no TLR agonist added.

tumor destruction; when therapy was initiated 3 days after B16 injection, tumors grew initially, but the combination therapy effected complete tumor regressions (data not shown). No toxicities of treatment were observed.

To analyze the mechanisms underlying these effects, we isolated tumor-infiltrating lymphocytes from wild-type B16 challenge sites. The coexpression of MFG-E8 resulted in increased intratumoral Tregs (Figure 8C), whereas the coexpression of the RGE mutant inhibited Treg recruitment compared with B16 cells secreting only GM-CSF (Figure 8D). The RGE mutant also enhanced the activation of CD8^{+} tumor-infiltrating lymphocytes and increased the numbers of MHC class I restricted, tyrosinase related protein 2–specific (TRP-2–specific) IFN-γ–secreting CD8^{+} effector cells, whereas these were decreased with MFG-E8 (Figure 8E).

Tumors harvested from treated mice showed striking differences in gross appearance. Melanomas arising in naive mice (data not shown) or in animals that received the GM-CSF/MFG-E8 vaccine were heavily pigmented, whereas those developing after the GM-CSF vaccine or the GM-CSF/RGE combination (prior to rejection) were not (Figure 8F). Histopathologic examination confirmed the variation in melanin production (data not shown). These findings suggest that MFG-E8–mediated modulation of immunity against melanocyte differentiation antigens, such as TRP-2, sculpts the phenotype of progressive tumors, consistent with the concept of

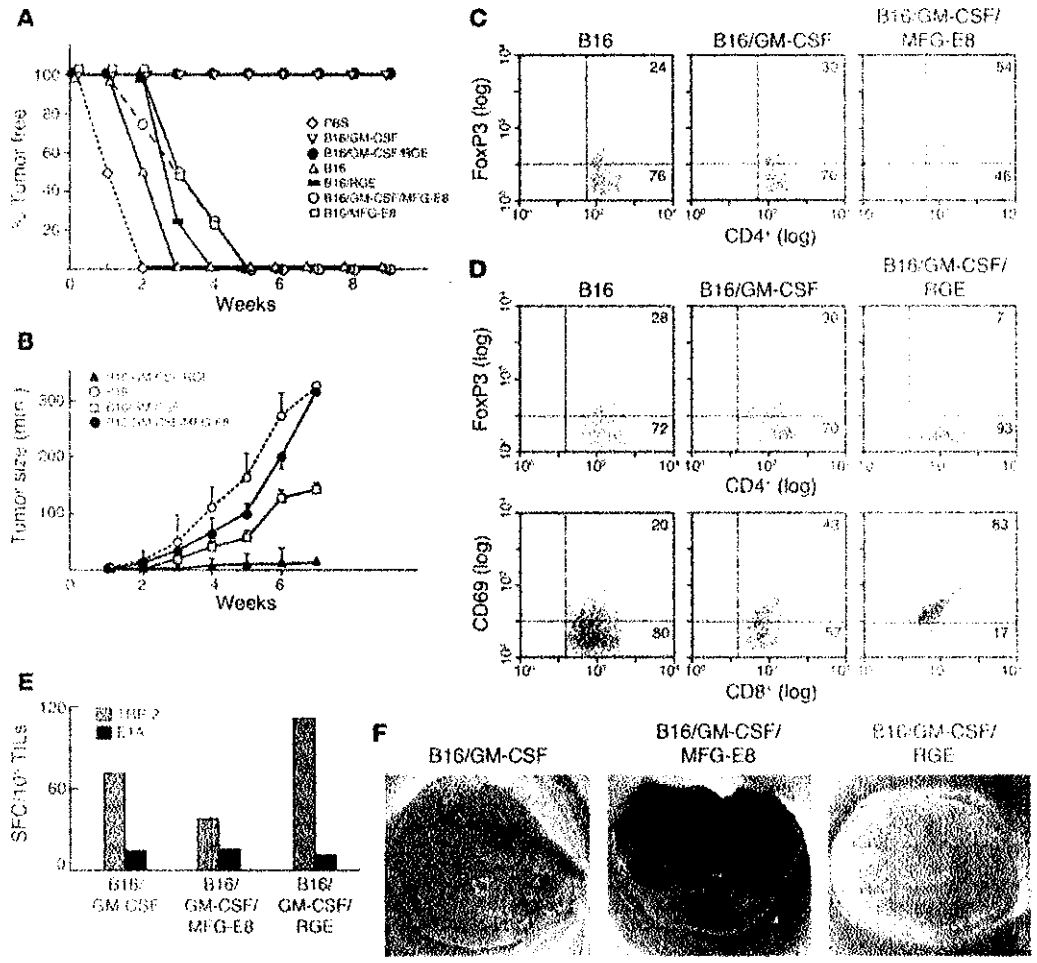

Figure 8

MFG-E8 modulates the vaccination activity of irradiated, GM-CSF–secreting B16 cells. (**A**) Wild-type C57BL/6 mice were vaccinated subcutaneously with 1×10^6 irradiated B16 cells as indicated and challenged on day 7 with 1×10^6 live B16 cells (8 mice per group). (**B**) C57BL/6 mice were injected with 1×10^6 live B16 cells, and the indicated vaccines were administered on days 0, 7, and 14 (8 mice per group). (**C**) Tumor-infiltrating lymphocytes were harvested from the B16 challenge sites of mice treated with the indicated vaccines and analyzed for FoxP3-expressing CD4⁺ T cells. Results are representative of 5 experiments. Numbers refer to the percentage of cells within an indicated gate. (**D**) Tumor-infiltrating lymphocytes were analyzed for FoxP3-expressing CD4⁺ T cells and CD8⁺ T cell activation. Similar results were obtained in 2 experiments. (**E**) Tumor-infiltrating lymphocytes were analyzed for TRP-2–specific IFN-γ production with an ELISPOT. E1A denotes a control H-2^b restricted peptide derived from the adenoviral E1A gene product. SFC, spot forming cells. (**F**). Gross appearance of B16 challenge tumors in mice treated with the indicated vaccines (8 mice for B16/GM-CSF and B16/GM-CSF/MFG-E8 and 2 mice for B16/GM-CSF/RGE).

immune editing (33). Furthermore, the complete tumor regressions effected by the GM-CSF/RGE combination likely involved a diversified host response that targeted multiple tumor-associated antigens in addition to melanocyte differentiation proteins.

Discussion

GM-CSF was initially characterized as a factor in lung conditioned medium that stimulated the growth of hematopoietic cells from bone marrow progenitors (34). Subsequent work established additional roles for the cytokine in enhancing resistance to tumors and infection and in modulating inflammatory pathology (35). In this study, we identified the phosphatidylserine-binding protein MFG-E8 as a major determinant of GM-CSF activities. Under steady-state conditions, GM-CSF induced MFG-E8 expression on antigen-presenting cells, resulting in the efficient phagocytosis of apoptotic cells, the maintenance of Tregs in the periphery, and the suppression of autoreactive Th1 and Th17 cells. However, under conditions of stress, TLR agonists or necrotic cells downregulated MFG-E8 expression whereby GM-CSF elicited protective responses through an MFG-E8–independent mechanism. Together, these findings elucidate a GM-CSF/MFG-E8 homeostatic circuit that regulates the balance of CD4⁺ T cell subsets (Figure 9).

The delineation of a GM-CSF/MFG-E8 pathway helps clarify the complex functions of this cytokine in inflammatory disease and cancer. Although GM-CSF deficiency alone is not sufficient to promote spontaneous tumor formation, loss of the cytokine precipitates a SLE-like disorder (12). The impaired uptake of apoptotic cells, which results in decreased Tregs and increased

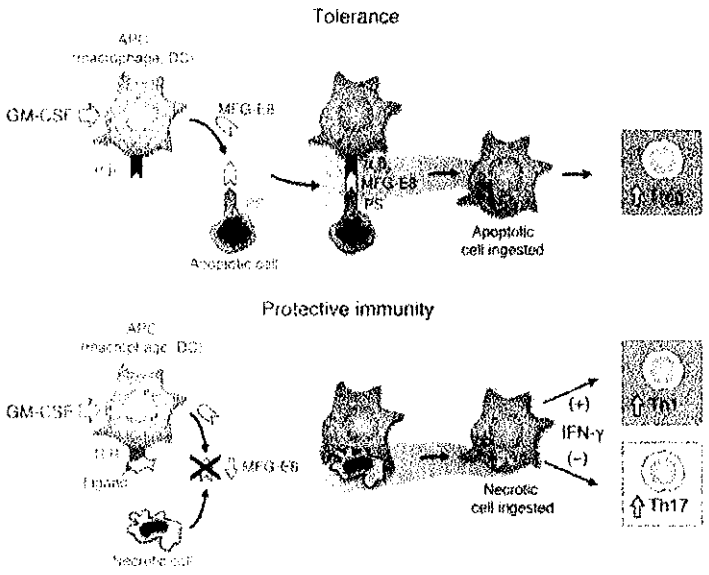

Figure 9
Proposed model of the dual roles for GM-CSF in tolerance and protective immunity. Top: GM-CSF is required for the expression of MFG-E8 in antigen-presenting cells. Upon exposure of phosphatidylserine (PS) on the surface of apoptotic cells, MFG-E8 promotes their engulfment by antigen-presenting cells, which results in the stimulations of Tregs. Bottom: In the setting of stress, TLR agonists and necrotic cells downregulate MFG-E8 on antigen-presenting cells, whereby GM-CSF promotes CD4+ effector T cell responses.

autoreactive Th1 cells, compromises tolerance and evokes the development of autoantibodies and immune complex mediated glomerulonephritis. In contrast, the concurrent ablation of IFN-γ triggers an expansion of autoreactive Th17 cells, with widespread acute and chronic granulomatous inflammation that resembles the pathology observed in IL-23 and IL-17 transgenic mice (36, 37). Furthermore, aged GM-CSF/IFN-γ compound deficient mice succumb at high frequency to hematologic and solid malignancies, consistent with a key role for the IL-23/IL-17 axis in inflammation-driven carcinogenesis (38, 39). IL-23 inhibits the intratumoral infiltration of cytotoxic T cells, whereas IL-17 engenders the production of angiogenic factors and matrix metalloproteinases that foster tumor formation. Our results therefore underscore the high carcinogenic potential of defective phagocytosis of apoptotic cells coupled with compromised IFN-γ signaling. Indeed, mutations in the genes encoding macrophage-scavenger receptor 1 and ribonuclease L, an endoribonuclease synthesized in response to interferon, are tumor susceptibility loci in humans (40).

While the loss of GM-CSF contributes to tumor promoting inflammation, paradoxically many tumors constitutively secrete low levels of the cytokine, which may be linked with disease progression (6). Previous work established that GM-CSF stimulates myeloid suppressor cells that antagonize cytotoxic lymphocytes through the elaboration of reactive nitrogen and oxygen species and the modulation of arginine metabolism (7, 41, 42). Our findings suggest that GM-CSF–induced MFG-E8 expression may be an additional mechanism of immune suppression in the tumor microenvironment. Consistent with this idea, tumor-associated macrophages in diverse human malignancies display high levels of MFG-E8 by immunohistochemistry (Supplemental Figure 6 and data not shown). The local release of TGF-β and CCL22 might foster the recruitment and maintenance of FoxP3+ Tregs that attenuate the cytotoxic activity of innate and adaptive antitumor effectors (43). Moreover, since angiogenesis induced by VEGF also requires MFG-E8 engagement of $\alpha_v\beta_5$ integrins on endothelial cells (44), the GM-CSF/MFG-E8 pathway might support tumor development in multiple ways.

In light of the immunoregulatory activities of MFG-E8, why does the provision of GM-CSF as cancer therapy increase antitumor responses? Our results indicate that diverse TLR agonists and necrotic cells downregulate the expression of MFG-E8 on antigen presenting cells, and this suppression may be required for protective immunity. Indeed, enforced production of MFG-E8 antagonized the immunostimulatory impact of TLR agonists and irradiated, GM-CSF–secreting tumor cell vaccines, whereas high levels of endogenous MFG-E8 were associated with tolerogenic dendritic cell subsets. While blockade of MFG-E8 function with the RGE mutant was insufficient to evoke protection in the B16 melanoma model, in conjunction with GM-CSF, the strategy proved therapeutic against preexisting lesions. This intensified activity suggests that irradiated GM-CSF–secreting tumor vaccines alone trigger only partial downregulation of MFG-E8 in vivo. The enhanced immunity elicited by the combination treatment involved the inhibition of Tregs and the amplification of $CD8^+$ cytotoxic T cells, resulting in a diversified host response capable of mediating regression of established tumors in the absence of toxicity. This mechanism of therapeutic synergy is distinct from that underlying CTLA-4 antibody blockade, which primarily targets effector cells but also increases Tregs (5). Thus, the 3 approaches together might prove complementary and achieve even higher levels of protective tumor immunity.

Methods

Mice. GM-CSF–, GM-CSF/IL-3–, IFN-γ– (45), and GM-CSF/IL-3/IFN-γ–deficient mice were backcrossed at least 9 generations onto the C57BL/6 strain and housed under specific pathogen–free conditions. Genotypes were confirmed by PCR (12), and the experiments were approved by the Association for Assessment and Accreditation of Laboratory Animal Care International–accredited Dana-Farber Cancer Institute Institutional Animal Care and Use Committee.

Phagocytosis assays. Wild-type thymocytes were exposed to 10 μM dexamethasone for 6 hours, splenocytes were exposed to 40 Gy γ-irradiation, and Jurkat T cells were exposed to 5 mg/ml etoposide for 16 hours to trigger apoptosis. Cell necrosis was induced with repetitive freeze-thaw cycles. Wild-type apoptotic or necrotic cells (scored with Annexin V and propidium iodide) were labeled with 5- (and 6-) carboxytetramethylrhodamine, succinimidyl ester (5[6]-TAMRA, SE; Invitrogen) or carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) as described in ref. 12.

Thioglycollate-elicited peritoneal macrophages or CD11b microbead purified (Miltenyi Biotec) tissue macrophages were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, and penicillin/streptomycin. Dendritic cells were isolated from spleens using CD11c microbeads (Miltenyi Biotec) or generated from bone marrow cells by culture for 7 days using conditioned media (added on days 0, 2, and 4)

from B16 cells secreting GM-CSF or Flt3-L (46). Macrophages or dendritic cells were exposed to labeled apoptotic or necrotic cells and evaluated for phagocytosis efficiency by flow cytometry as previously reported (12).

Cytokine assays. TGF-β, IL-6, IL-1β, IL-12p70, IL-23p19, IL-10, and TNF-α were measured in culture supernatants by ELISA, according to the manufacturer's directions (Pierce Biotechnology, R&D Systems, eBioscience). For intracellular cytokine staining, T cells were stimulated with 1 mg/ml PMA and 50 ng/ml ionomycin; treated with GolgiPlug (BD Biosciences — Pharmingen); stained with anti-CD3, anti-CD4, or anti-CD8 mAbs; fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences — Pharmingen); stained again with anti–IFN-γ, anti–IL-17, and isotype control mAbs (BD Pharmingen); and analyzed by flow cytometry.

Histology and immunohistochemistry. Formalin-fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissue sections were treated for antigen retrieval with a pressure cooker for 20 minutes and then incubated with 5 μg/ml of primary antibodies or a corresponding IgG fraction of preimmune serum in 3% BSA/PBS blocking solution for 16 hours at 4°C. The hamster anti-mouse MFG-E8 Ab (clone 18A-G10; MBL International) and mouse anti-human MFG-E8 Ab (clone 278918; R&D Systems) were used. The primary antibodies were then visualized with the corresponding secondary biotinylated antibody and the streptavidin-peroxidase complex from Vector Laboratories.

Cell proliferation assays. Peritoneal macrophages were exposed to apoptotic or necrotic cells for 2 hours, washed, cocultured with Balb/c splenocytes for 72 hours, and ^{3}H-thymidine uptake was determined. For suppression assays, CD4$^+$CD25$^+$ T cells were isolated from the splenocytes of wild-type and mutant mice with the regulatory T cell isolation kit (Miltenyi Biotec) following the manufacturer's instructions. Purified wild-type CD4$^+$CD25$^-$ spleme effector T cells were activated with 1 μg/ml plate-bound anti-CD3 mAb (clone 2C11; BD Biosciences — Pharmingen) in the presence or absence of regulatory CD4$^+$CD25$^+$ T cells at various ratios for 72 hours, and proliferation was measured with ^{3}H-thymidine incorporation. The suppressor activity was calculated as the percentage of proliferation measured in the cocultures versus effector cells only.

Chemotaxis assays. Supernatants of macrophages exposed to apoptotic cells were diluted 1:10 in culture media and added to the bottom wells of a microchamber containing an 8-μm pore polycarbonate filter (Costar-Corning). CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells were applied to the upper chamber in the presence or absence of anti-human CCL22/MDC neutralizing Ab or isotype control (R&D Systems), and the cells migrating to the bottom chamber were counted.

Flow cytometry. Macrophages were pretreated with GolgiPlug (BD Biosciences — Pharmingen), stained with anti-CD11b mAb (BD Biosciences — Pharmingen), fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences — Pharmingen), and stained again with unconjugated MFG-E8 mAb (Alexia) or Gas6 mAb (R&D Systems) followed by PE-labeled goat anti-IgG2 Ab (BD Biosciences — Pharmingen). For FoxP3 staining, lymphoid cells were labeled with anti-CD3 and CD4 mAbs (BD Biosciences — Pharmingen), washed, and then stained with PE-labeled anti-FoxP3 Ab using the FoxP3 staining set according to the manufacturer's protocol (eBioscience). Cell acquisition was performed with a FW501 flow cytometer (Beckman Coulter) and analyzed by FlowJo software (version 6.3.1; Tree Star).

Fluorescence microscopy. A total of 1×10^5 purified wild-type or mutant macrophages were stained with the green fluorescent cell linker reagent (Sigma-Aldrich), while 1×10^5 dexamethasone-treated wild-type thymocytes were stained with the red fluorescent cell linker reagent (Sigma-Aldrich) according to the manufacturer's instructions. Apoptotic thymocytes were cocultured with macrophages for 6 hours on glass slides. The samples were then washed 3 times to remove floating cells, fixed with 20% methanol at –20°C for 5 minutes, and visualized using a TE2000-U inverted fluorescence microscope (Nikon).

Retroviral mediated gene transfer. Full-length sequences encoding the open reading frames of MFG-E8 or the RGE mutant (which replaces the RGD sequence in the second EGF domain with RGE) were introduced into the pMFG retroviral vector and high-titer VSV-G–pseudotyped replication-defective viral stocks were prepared with 293-GPG cells as previously described (46). Peritoneal macrophages were induced to replicate by culture in DMEM plus 10% fetal calf serum supplemented with 10 ng/ml M-CSF (R&D Systems). Viral supernatants were added to the cultured macrophages overnight in the presence of polybrene (8 μg/ml) to facilitate infection. The transduced cells were then washed and used for experiments 2–3 days later.

After 48 hours of preconditioning with 5-fluorouracil (150 mg/kg), bone marrow cells were isolated from 8- to 10-week-old wild-type and GM-CSF/IL-3/IFN-γ–deficient mice and cultured overnight with X-VIVO (Cambrex) supplemented with stem cell factor (100 ng/ml) and thrombopoietin (50 ng/ml). The cells were transduced with retroviral supernatants for 48 hours and then 1×10^6 cells were injected into lethally irradiated recipients (2 doses of 5.6 Gy, 6 hours apart, using a ^{137}Cs source). Eight weeks after transplant, mice were sacrificed and analyzed for MFG-E8 expression, phagocytic capacity, and CD4$^+$ T cell subsets as above.

B16 melanoma experiments. For tumor prevention studies, 8- to 12-week-old female C57BL/6 mice were injected subcutaneously in the flank with 1×10^6 irradiated (150 Gy) wild-type or retrovirally transduced (GM-CSF, MFG-E8, and/or RGE) B16 cells and 7 days later challenged subcutaneously on the back with 1×10^6 live B16 cells. For the therapy model, mice were injected on day 0 or 3 with 5×10^5 live B16 cells and treated on days 0, 7, and 14 with 1×10^6 irradiated, engineered B16 cells. Tumor growth was monitored, and the product of tumor diameters was recorded.

Tumor-infiltrating lymphocytes were obtained from B16 challenge sites using a Nocoprep (Axis-Shield) cell gradient separation followed by CD3$^+$ T cell purification with anti-CD3–labeled magnetic beads (Miltenyi Biotec). The cells were analyzed by flow cytometry using mAbs against CD4, CD8, CD69, and FoxP3. Antigen-specific CD8$^+$ responses against H-2^b restricted peptides derived from TRP-2 (180–188: SVYDFFVWL) or E1A (234–243: SGPSNTPPEI) were determined by incubating lymphocytes for 72 hours with 1×10^5 B16 cells and 25 U/ml IL-2 and measuring IFN-γ production by ELISPOT using peptide-pulsed splenocytes as targets.

Statistics. A 1-sided exact Wilcoxon test was employed for statistical analysis.

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Info on Toxin(s)

Gemcitabine Hydrochloride for Injection

Eli Lilly and Company
Material Safety Data Sheet

Per conversation with PI Sept 15, 2011
amount used at once: 125 ug / 20g mouse (20g mouse)
LD_{50} (mg/Kg, mouse): >333
amount on hand: 50 mg

Section 1 - Chem

Manufacturer:
Eli Lilly and Company
Lilly Corporate Center
Indianapolis, IN 46285

Common Name: Gemcitabine Hydrochloride for Injection

Chemical Name: Cytidine, 2'-deoxy-2',2'-difluoro-, monohydrochloride
Chemical Name 2: 2'-Deoxy-2',2'-difluorocytidine monohydrochloride (beta-isomer)
Synonym(s): Gemcitabine HCl formulation; Gemcitabine; 264368 formulation; 188011 hydrochloride formulation
Trademarks(s): Gemcin; Gemtro; Gemzar
Lilly Item Code(s): VL7501; VL7502; VL7538; VL7540

See attached glossary for abbreviations.

Section 2 - Composition / Information on Ingredients

Ingredient	CAS	Concentration %
Gemcitabine Hydrochloride	122111-03-9	51 - 53
Excipients	NA	47 - 49

Contains no hazardous components (one percent or greater) or carcinogens (one-tenth percent or greater) not listed above.

Exposure Guidelines:
Gemcitabine hydrochloride - LEG 0.3 micrograms/m3 TWA for 8 hours, LEG 0.2 micrograms/m3 TWA for 12 hours. Excursion guidance is to maintain full shift TWA.

Section 3 - Hazards Identification

Appearance: White to off-white powder or freeze-dried plug
Physical State: Solid
Odor: Odorless

Emergency Overview

Special
R = Reproductive

Emergency Overview Effective Date: 29-Oct-1998

Lilly Laboratory Labeling Codes:
Health 2 **Fire** 1 **Reactivity** 0 **Special** R

Primary Physical and Health Hazards: Skin Permeable. Mutagen. Irritant (eyes, skin). Reproductive and Blood Effects.

Caution Statement: Gemcitabine Hydrochloride for Injection contains gemcitabine hydrochloride which may enter the body through the skin, alters genetic material, and may be irritating to the eyes and skin. Effects of exposure may include decreased fertility, fetal changes, and decreased blood cell counts.

Routes of Entry: Inhalation and skin absorption.

Effects of Overexposure: Based on animal data, gemcitabine hydrochloride for injection may be absorbed through the skin in amounts capable of producing systemic toxicity and may be irritating to the eyes and skin. Effects of exposure due to therapeutic use may include, but are not limited to, decreased blood cell counts, nausea, vomiting, edema, rash, elevated liver enzymes, and flu-like syndrome.

Medical Conditions Aggravated by Exposure: None known.

Carcinogenicity: No carcinogenicity data found. Not listed by IARC, NTP, ACGIH, or OSHA.

Section 4 - First Aid Measures

Eyes: Hold eyelids open and flush with a steady, gentle stream of water for 15 minutes. See an ophthalmologist (eye doctor) or other physician immediately.

Skin: Remove contaminated clothing and clean before reuse. Wash all exposed areas of skin with plenty of soap and water. Get medical attention if irritation develops.

Inhalation: Move individual to fresh air. Get medical attention if breathing difficulty occurs. If not breathing, provide artificial respiration assistance (mouth-to-mouth) and call a physician immediately.

Ingestion: Do not induce vomiting. Call a physician or poison control center. If available, administer activated charcoal (6-8 heaping teaspoons) with two to three glasses of water. Do not give anything by mouth to an unconscious person. Immediately transport to a medical care facility and see a physician.

Section 5 - Fire Fighting Measures

Flash Point: No applicable information found
UEL: No applicable information found
LEL: No applicable information found

Extinguishing Media: Use water, carbon dioxide, dry chemical, foam, or Halon.

Unusual Fire and Explosion Hazards: As a finely divided material, may form dust mixtures in air which could explode if subjected to an ignition source.

Hazardous Combustion Products: May emit toxic chloride and fluoride fumes when exposed to heat or fire.

Section 6 - Accidental Release Measures

Spills: Use double pairs of latex disposable gloves which must be disposed of within an hour, goggles, impermeable body covering, and approved HEPA-filtered or supplied-air respirator. If material spills occur in production area, use either wet clean-up methods, ensuring that no airborne dusts or aerosols are formed, or appropriate vacuum cleaners having high efficiency particulate air (HEPA) filters.

It is recommended that areas handling final finished product have cytotoxic spill kits available. Spill kits should include impermeable body covering, shoe covers, latex and utility latex gloves, goggles, approved HEPA respirator, disposable dust pan and scoop, absorbent towels, spill control pillows, disposable sponges, sharps container, disposable garbage bag, and a hazardous waste label.

Section 7 - Handling and Storage

Storage Conditions: Controlled Room Temperature: 15 to 30 C (59 to 86 F).

Section 8 - Exposure Controls / Personal Protection

See Section 2 for Exposure Guideline information.

For appropriate handling precautions in specific laboratory, manufacturing, or clinical health care operations, consult with a health and safety or technical services representative.

In clinical health care settings, follow OSHA Technical Manual, Section VI, Chapter 2 - Controlling Occupational Exposure to Hazardous Drugs. This chapter covers protection of employees during cytotoxic drug preparation, administration, disposal, and the handling of human waste products potentially contaminated with cytotoxic drug substances.

GENERAL: For all work environments, wear eye protection, avoid skin contact, wear gloves, and take other appropriate precautions.

Respiratory Protection: When the exposure guidelines may be exceeded, use an approved HEPA-

filtered or supplied-air respirator.

Eye Protection: Chemical goggles and/or face shield.

Ventilation: Extensive local exhaust, ventilated enclosure (HEPA-filtered balance enclosure, fume hood, or Class II or III vertical flow biosafety cabinet), or enclosed process equipment.

Other Protective Equipment: Chemical-resistant gloves and impermeable body covering to minimize skin contact. If handled in a ventilated enclosure, as in a laboratory setting, respirator and goggles or face shield may not be required. Safety glasses are always required.

Additional Exposure Precautions: In production settings, airline-supplied, hood-type respirators are preferred. Shower and change clothing if skin contact occurs.

Section 9 - Physical and Chemical Properties

Appearance: White to off-white powder or freeze-dried plug
Odor: Odorless
Boiling Point: Not applicable
Melting Point: No applicable information found
Density: No applicable information found
pH: Acidic
Evaporation Rate: No applicable information found
Water Solubility: Soluble
Vapor Density: No applicable information found
Vapor Pressure: No applicable information found

Section 10 - Stability and Reactivity

Stability: Stable at normal temperatures and pressures.

Incompatibility: May react with strong oxidizing agents (e.g., peroxides, permanganates, nitric acid, etc.).

Hazardous Decomposition: May emit toxic fluoride and chloride fumes when heated to decomposition.

Hazardous Polymerization: Will not occur.

Section 11 - Toxicological Information

Acute Exposure

No data available for mixture or formulation. Data for ingredient(s) or related material(s) are presented.

Oral:

Gemcitabine hydrochloride - Rat, 500 mg/kg, no deaths.
Mouse, mortality due to intestinal lesions reported when given a single dose of 333 mg/kg and higher.

Skin:
Gemcitabine hydrochloride for injection - Rabbit, median lethal dose estimated greater than 1000 mg/kg, mortality, reduced activity, diarrhea, weight loss, few feces, pale eyes, salivation.

Inhalation: No applicable information found.

Skin Contact:
Gemcitabine hydrochloride for injection - Rabbit, irritant

Eye Contact:
Gemcitabine hydrochloride - Rabbit, irritant

Chronic Exposure

No data available for mixture or formulation. Data for ingredient(s) or related material(s) are presented.

Target Organ Effects:
Gemcitabine hydrochloride - Blood effects (decreased red blood cell, white blood cell, and platelet counts).

Reproduction:
Gemcitabine hydrochloride - Decreased sperm formation and decreased fertility in males, and reproductive tissue changes. Depressed fetal viability and weight and malformations at doses toxic to the mother.

Sensitization:
Gemcitabine hydrochloride - Guinea pig, subcutaneous, negative systemic response.

Mutagenicity:
Gemcitabine hydrochloride - Mutagenic in mouse lymphoma assay and mouse micronucleus test. Not mutagenic in bacterial cells and other mammalian cell tests.

Section 12 - Ecological Information

No environmental data for the mixture or formulation. The environmental information for ingredient(s) or related material(s) are presented.

Ecotoxicity Data:
Gemcitabine
Rainbow trout 96-hour median lethal concentration: > 1043 mg/L
Fathead minnow 96-hour median lethal concentration: > 1014 mg/L
Daphnia magna 48-hour median effective concentration: > 999 mg/L
Green algae (S. capricornutum) median effective concentration: 5.4 mg/L (average specific growth rate)
Microorganisms
fungus (Chaetomium globosum): MIC > 1000 mg/L

mold (Aspergillus flavus): MIC > 1000 mg/L
soil bacteria (Comamonas acidovorans): MIC > 1000 mg/L
N-fixing bacteria (Azotobacter chroococcum): MIC > 1000 mg/L
blue-green algae (Nostoc sp.): MIC 800 mg/L

Environmental Fate:
Gemcitabine hydrochloride
Dissociation constant (pKa): 3.58
Kow: 0.053, 0.053, 0.052 (pH 5, 7, 9)
Solubility (g/L): 16.0, 15.3, 15.8 (pH 5, 7, 9)
Light absorption (nm): 268 - 269
Hydrolysis: no significant hydroylsis
Aerobic biodegradation half-life: 30% in 28 days

Environmental Summary:
Gemcitabine - Practically non-toxic to fish and microorganisms and moderately toxic to green algae. Low potential to bioaccumulate in aquatic organisms. Expected to be persistent in the environment based on slow rates of hydrolysis and biodegradation.

Lilly Aquatic Exposure Guideline (LAEG):
Gemcitabine hydrochloride
LAEG for Drinking Water: 0.045 micrograms/L
LAEG for Chronic Exposure: 1.0 micrograms/L
LAEG for Acute Exposure of Aquatic Organisms: 5400 micrograms/L

Section 13 - Disposal Considerations

Waste Disposal: To avoid accidental exposure due to waste handling, place waste residue in a segregated, sealed plastic container. Used syringes, needles, and sharps should not be crushed, clipped, or recapped, but placed directly into an approved sharps container. Dispose of any cleanup materials and waste residue according to all applicable laws and regulations, e.g., secure chemical landfill disposal.

Section 14 - Transport Information

Regulatory Organizations:

DOT: Not Regulated

ICAO/IATA: Not Regulated

IMO: Not Regulated

Section 15 - Regulatory Information

Below is selected regulatory information chosen primarily for possible Eli Lilly and Company usage. This section is not a complete analysis or reference to all applicable regulatory

information. Please consider all applicable laws and regulations for your country/state.

U.S. Regulations

Gemcitabine hydrochloride
TSCA - No
CERCLA - Not on this list
SARA 302 - Not on this list
SARA 313 - Not on this list
OSHA Substance Specific - No

EU Regulations

EC Classification
Contains gemcitabine hydrochloride (C = 51 to 53%)
Xn (Harmful)
Xi (Irritant)
Reproductive Category 3
Mutagen Category 2

Risk Phrases
R 21 - Harmful in contact with skin.
R 36/38 - Irritating to eyes and skin.
R 62 - Possible risk of impaired fertility.
R 63 - Possible risk of harm to the unborn child.
R 46 - May cause heritable genetic damage.

Safety Phrases
S 25 - Avoid contact with eyes.
S 26 - In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S 36/37 - Wear suitable protective clothing and gloves.
S 53 - Avoid exposure - obtain special instructions before use.

Section 16 - Other Information

MSDS Sections Revised: Section 2.

As of the date of issuance, we are providing available information relevant to the handling of this material in the workplace. All information contained herein is offered with the good faith belief that it is accurate. THIS MATERIAL SAFETY DATA SHEET SHALL NOT BE DEEMED TO CREATE ANY WARRANTY OF ANY KIND (INCLUDING WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE). In the event of an adverse incident associated with this material, this safety data sheet is not intended to be a substitute for consultation with appropriately trained personnel. Nor is this safety data sheet intended to be a substitute for product literature which may accompany the finished product.

For additional information contact:
Eli Lilly and Company
Hazard Communication

317-277-6029

For additional copies contact:
Eli Lilly and Company
1-800-LILLY-Rx (1-800-545-5979)

GLOSSARY:

ACGIH = American Conference of Governmental Industrial Hygienists
AIHA = American Industrial Hygiene Association
BEI = Biological Exposure Index
CAS Number = Chemical Abstract Service Registry Number
CERCLA = Comprehensive Environmental Response Compensation and Liability Act (of 1980)
CHAN = Chemical Hazard Alert Notice
CHEMTREC = Chemical Transportation Emergency Center
DOT = Department of Transportation
EC = European Community
EINECS = European Inventory of Existing Chemical Substances
ELINCS = European List of New Chemical Substances
EPA = Environmental Protection Agency
HEPA = High Efficiency Particulate Air (Filter)
IARC = International Agency for Research on Cancer
ICAO/IATA = International Civil Aviation Organization/International Air Transport Association
IEG = Lilly Interim Exposure Guideline
IMO = International Maritime Organization
Kow = Octanol/Water Partition Coefficient
LEG = Lilly Exposure Guideline
LEL = Lower Explosive Limit
MSDS = Material Safety Data Sheet
MSHA = Mine Safety and Health Administration
NA = Not Applicable, except in Section 14 where NA = North America
NADA = New Animal Drug Application
NAIF = No Applicable Information Found
NCI = National Cancer Institute
NIOSH = National Institute for Occupational Safety and Health
NOS = Not Otherwise Specified
NTP = National Toxicology Program
OSHA = Occupational Safety and Health Administration
PEL = Permissible Exposure Limit (OSHA)
RCRA = Resource Conservation and Recovery Act
RQ = Reportable Quantity
RTECS = Registry of Toxic Effects of Chemical Substances
SARA = Superfund Amendments and Reauthorization Act
STEG = Lilly Short Term Exposure Guideline
STEL = Short Term Exposure Limit
TLV = Threshold Limit Value (ACGIH)
TPQ = Threshold Planning Quantity
TSCA = Toxic Substances Control Act
TWA = Time Weighted Average/8 Hours Unless Otherwise Noted
UEL = Upper Explosive Limit
UN = United Nations

WEEL = Workplace Environmental Exposure Level (AIHA)

Safety data sheet
according to 1907/2006/EC, Article 31

Printing date 03.02.2011 Revision: 03.02.2011

1 Identification of the substance/mixture and of the company/undertaking

· **Product identifier**

· **Trade name:** Tunicamycin

· **Article number:** BML-CC104
· **CAS Number:**
11089-65-9
· **Relevant identified uses of the substance or mixture and uses advised against**
· **Application of the substance / the preparation:** Laboratory chemicals

· **Details of the supplier of the safety data sheet**
· **Manufacturer/Supplier:**
Enzo Life Sciences International, Inc.
10 Executive Boulevard
Farmingdale, NY 11735
U.S.A.
msds@enzolifesciences.com

· **Further information obtainable from:** Customer service
· **Emergency telephone number:** During normal opening times: +1 610-941-0430

2 Hazards identification

· **Classification of the substance or mixture**
· **Classification according to Regulation (EC) No 1272/2008**


GHS06 skull and crossbones

Acute Tox. 2 H300 Fatal if swallowed.
Acute Tox. 1 H330 Fatal if inhaled.


GHS08 health hazard

STOT RE 2 H373 May cause damage to organs through prolonged or repeated exposure.

· **Classification according to Directive 67/548/EEC or Directive 1999/45/EC:**

T+; Very toxic

R26/28: Very toxic by inhalation and if swallowed.

R33: Danger of cumulative effects.
· **Information concerning particular hazards for human and environment:** Not applicable.

· **Label elements**
· **Labelling according to Regulation (EC) No 1272/2008:**
The substance is classified and labelled according to the CLP regulation.
· **Hazard pictograms:**


GHS06


GHS08

· **Signal word:** Danger
· **Hazard statements:**
H300 Fatal if swallowed.
H330 Fatal if inhaled.
H373 May cause damage to organs through prolonged or repeated exposure.

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Trade name: Tunicamycin

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· **Precautionary statements**
P260 Do not breathe dust/fume/gas/mist/vapours/spray.
P301+P310 IF SWALLOWED: Immediately call a POISON CENTER or doctor/physician.
P310 Immediately call a POISON CENTER or doctor/physician.
P320 Specific treatment is urgent (see on this label).
P405 Store locked up.
P501 Dispose of contents/container in accordance with local/regional/national/international regulations.
· **Other hazards**
· **Results of PBT and vPvB assessment**
· **PBT:** Not applicable.
· **vPvB:** Not applicable.

3 Composition/information on ingredients

· **Chemical characterization: Substances**
· **CAS No. Description:**
11089-65-9 Tunicamycin
· **Identification number(s):** Not applicable

4 First aid measures

· **Description of first aid measures**
· **General information:**
Immediately remove any clothing soiled by the product.
Remove breathing equipment only after contaminated clothing have been completely removed.
In case of irregular breathing or respiratory arrest provide artificial respiration.
· **After inhalation:**
Supply fresh air or oxygen; call for doctor.
In case of unconsciousness place patient stably in side position for transportation.
· **After skin contact:**
Immediately wash with water and soap and rinse thoroughly.
Remove contaminated clothing.
· **After eye contact:**
Rinse opened eye for several minutes under running water. Then consult a doctor.
· **After ingestion:** Do not induce vomiting; call for medical help immediately.
· **Information for doctor:**
· **Most important symptoms and effects, both acute and delayed**
No further relevant information available.
· **Indication of any immediate medical attention and special treatment needed**
No further relevant information available.

5 Firefighting measures

· **Extinguishing media**
· **Suitable extinguishing agents:**
CO2, powder or water spray. Fight larger fires with water spray or alcohol resistant foam.
· **Special hazards arising from the substance or mixture** No further relevant information available.
· **Advice for firefighters**
· **Protective equipment:**
Mouth respiratory protective device.
Wear self-contained respiratory protective device.
Do not inhale explosion gases or combustion gases.

(Contd. on page 3)

Safety data sheet
according to 1907/2006/EC, Article 31

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Trade name: Tunicamycin

(Contd. of page 2)

Wear fully protective suit.

6 Accidental release measures

· **Personal precautions, protective equipment and emergency procedures:**
Avoid formation of dust.
Wear protective clothing.
· **Environmental precautions:** Do not allow to enter sewers/ surface or ground water.
· **Methods and material for containment and cleaning up:**
Dispose contaminated material as waste according to item 13.
Ensure adequate ventilation.
· **Reference to other sections**
See Section 7 for information on safe handling.
See Section 8 for information on personal protection equipment.
See Section 13 for disposal information.

7 Handling and storage

· **Handling:**
· **Precautions for safe handling:**
Thorough dedusting.
Ensure good ventilation/exhaustion at the workplace.
Open and handle receptacle with care.
· **Information about fire - and explosion protection:** Keep respiratory protective device available.

· **Conditions for safe storage, including any incompatibilities**
· **Storage:**
· **Requirements to be met by storerooms and receptacles:**
Store tightly sealed in a cool, dry and well ventilated location (see label for storage temperature and additional specific information).
· **Information about storage in one common storage facility:**
Keep away from heat, sources of ignition and incompatibles such as oxidizing agents.
· **Further information about storage conditions:**
Keep container tightly sealed.
Store under lock and key and with access restricted to technical experts or their assistants only.
· **Specific end use(s)** No further relevant information available.

8 Exposure controls/personal protection

· **Additional information about design of technical facilities:** No further data; see item 7.

· **Control parameters**
· **Ingredients with limit values that require monitoring at the workplace:** Not required.
· **Additional information:** The lists valid during the making were used as basis.

· **Exposure controls**
· **Personal protective equipment:**
· **General protective and hygienic measures:**
Keep away from foodstuffs, beverages and feed.
Immediately remove all soiled and contaminated clothing
Wash hands before breaks and at the end of work.
Store protective clothing separately.
· **Respiratory protection:**
In case of brief exposure or low pollution use respiratory filter device. In case of intensive or longer exposure use self-contained respiratory protective device.

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Trade name: Tunicamycin

(Contd. of page 3)

· **Protection of hands:**

Protective gloves

The glove material has to be impermeable and resistant to the product/ the substance/ the preparation.
Due to missing tests no recommendation to the glove material can be given for the product/the preparation/the chemical mixture.
Selection of the glove material on consideration of the penetration times, rates of diffusion and the degradation

· **Material of gloves**
The selection of the suitable gloves does not only depend on the material, but also on further marks of quality and varies from manufacturer to manufacturer.

· **Penetration time of glove material**
The exact break through time has to be found out by the manufacturer of the protective gloves and has to be observed.

· **Eye protection:** Not required.

9 Physical and chemical properties

· **Information on basic physical and chemical properties**	
· **General Information**	
· **Appearance:**	
Form:	Powder
Colour:	Off-white
· **Odour:**	Characteristic
· **Odour threshold:**	Not determined.
· **pH-value:**	Not applicable.
· **Change in condition**	
Melting point/Melting range:	Undetermined.
Boiling point/Boiling range:	Undetermined.
· **Flash point:**	Not applicable.
· **Flammability (solid, gaseous):**	Product is not flammable.
· **Ignition temperature:**	
Decomposition temperature:	Not determined.
· **Self-igniting:**	Not determined.
· **Danger of explosion:**	Product does not present an explosion hazard.
· **Explosion limits:**	
Lower:	Not determined.
Upper:	Not determined.
· **Vapour pressure:**	Not applicable.
· **Density:**	Not determined.
· **Relative density**	Not determined.
· **Vapour density**	Not applicable.
· **Evaporation rate**	Not applicable.
· **Solubility in / Miscibility with water:**	Not determined.
· **Segregation coefficient (n-octanol/water):**	Not determined.

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Trade name: Tunicamycin

(Contd. of page 4)

· **Viscosity:**
Dynamic: Not applicable.
Kinematic: Not applicable.
· **Other information** No further relevant information available.

10 Stability and reactivity

· **Reactivity**
· **Chemical stability:** Stable. Avoid strong oxidizing agents.
· **Thermal decomposition / conditions to be avoided:**
No decomposition if used according to specifications.
· **Possibility of hazardous reactions:** No dangerous reactions known.
· **Conditions to avoid:** No further relevant information available.
· **Incompatible materials:** No further relevant information available.
· **Hazardous decomposition products:** No dangerous decomposition products known.

11 Toxicological information

· **Information on toxicological effects**
· **Acute toxicity:**

· **LD/LC50 values relevant for classification:**	
LD50	>0,4 mg/kg (guinea pig) (subcutaneous)
	>0,2 mg/kg (rat) (subcutaneous)

· **Primary irritant effect:**
· **on the skin:** Not determined
· **on the eye:** Not determined
· **Sensitization:** No sensitizing effects known.

12 Ecological information

· **Toxicity**
· **Aquatic toxicity:** No further relevant information available.
· **Persistence and degradability:** No further relevant information available.
· **Behaviour in environmental systems:**
· **Bioaccumulative potential:** No further relevant information available.
· **Mobility in soil:** No further relevant information available.
· **Additional ecological information:**
· **General notes:**
Water hazard class 3 (German Regulation) (Assessment by list): extremely hazardous for water
Do not allow product to reach ground water, water course or sewage system, even in small quantities.
Danger to drinking water if even extremely small quantities leak into the ground.
· **Results of PBT and vPvB assessment**
· **PBT:** Not applicable.
· **vPvB:** Not applicable.
· **Other adverse effects** No further relevant information available.

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Trade name: Tunicamycin

(Contd. of page 5)

13 Disposal considerations

· **Waste treatment methods**
· **Recommendation:**
Must not be disposed together with household garbage. Do not allow product to reach sewage system.

· **Uncleaned packaging:**
· **Recommendation:** Disposal must be made according to official regulations.

14 Transport information

· **Maritime transport IMDG:**
· **Marine pollutant:** No
· **Special precautions for user:** Not applicable.
· **Transport in bulk according to Annex II of MARPOL73/78 and the IBC Code:** Not applicable.

15 Regulatory information

· **Chemical safety assessment:** A Chemical Safety Assessment has not been carried out.

16 Other information

This information is based on our present knowledge. However, this shall not constitute a guarantee for any specific product features and shall not establish a legally valid contractual relationship. For research use only. Not for drug, household or other uses.

· **Department issuing MSDS:** Customer service
· **Contact:** tel. +1 610-941-0430
· **Abbreviations and acronyms:**
IMDG: International Maritime Code for Dangerous Goods
GHS: Globally Harmonized System of Classification and Labelling of Chemicals
CAS: Chemical Abstracts Service (division of the American Chemical Society)
LC50: Lethal concentration, 50 percent
LD50: Lethal dose, 50 percent

Safety data sheet
according to 1907/2006/EC, Article 31

Printing date 04.03.2011 Revision: 04.03.2011

Trade name: Cycloheximide

(Contd. of page 1)


N; Dangerous for the environment

R51/53: Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

Muta. Cat. 3

· **Information concerning particular hazards for human and environment:** Not applicable.

· **Label elements**

· **Labelling according to Regulation (EC) No 1272/2008:**
The substance is classified and labelled according to the CLP regulation.

· **Hazard pictograms:**


GHS06 GHS08 GHS09

· **Signal word:** Danger

· **Hazard statements:**
H300 Fatal if swallowed.
H341 Suspected of causing genetic defects.
H360D May damage the unborn child.
H411 Toxic to aquatic life with long lasting effects.

· **Precautionary statements**
P281 Use personal protective equipment as required.
P273 Avoid release to the environment.
P301+P310 IF SWALLOWED: Immediately call a POISON CENTER or doctor/physician.
P321 Specific treatment (see on this label).
P405 Store locked up.
P501 Dispose of contents/container in accordance with local/regional/national/international regulations.

· **Other hazards**

· **Results of PBT and vPvB assessment**

· **PBT:** Not applicable.

· **vPvB:** Not applicable.

3 Composition/information on ingredients

· **Chemical characterization: Substances**

· **CAS No. Description:**
66-81-9 Cycloheximide

· **Identification number(s):**

· **EINECS Number:** 200-636-0

· **Index number:** 613-140-00-8

4 First aid measures

· **Description of first aid measures**

· **General information:**
Immediately remove any clothing soiled by the product.
In case of irregular breathing or respiratory arrest provide artificial respiration.

· **After inhalation:**
In case of unconsciousness place patient stably in side position for transportation.

· **After skin contact:**
Immediately wash with water and soap and rinse thoroughly.
Remove contaminated clothing.

(Contd. on page 3)

Safety data sheet
according to 1907/2006/EC, Article 31

Printing date 04.03.2011 Revision: 04.03.2011

1 Identification of the substance/mixture and of the company/undertaking

· **Product identifier**

· **Trade name:** Cycloheximide
· **Synonyms:**
Actidione, Naramycin A, 3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide

· **Article number:** ALX-380-269
· **CAS Number:**
66-81-9
· **EINECS Number:**
200-636-0
· **Index number:**
613-140-00-8
· **Relevant identified uses of the substance or mixture and uses advised against**
· **Application of the substance / the preparation:** Laboratory chemicals

· **Details of the supplier of the safety data sheet**
· **Manufacturer/Supplier:**
Enzo Life Sciences International, Inc.
10 Executive Boulevard
Farmingdale, NY 11735
U.S.A.
msds@enzolifesciences.com

· **Further information obtainable from:** Customer service
· **Emergency telephone number:** During normal opening times: +1 610-941-0430

2 Hazards identification

· **Classification of the substance or mixture**
· **Classification according to Regulation (EC) No 1272/2008**


GHS06 skull and crossbones

Acute Tox. 2 H300 Fatal if swallowed.


GHS08 health hazard

Muta. 2 H341 Suspected of causing genetic defects.
Repr. 1B H360D May damage the unborn child.


GHS09 environment

Aquatic Chronic 2 H411 Toxic to aquatic life with long lasting effects.

· **Classification according to Directive 67/548/EEC or Directive 1999/45/EC:**

T+; Very toxic

R28: Very toxic if swallowed.


T; Toxic
Repr. Cat. 2
R61: May cause harm to the unborn child.


Xn; Harmful

R40-68: Limited evidence of a carcinogenic effect. Possible risk of irreversible effects.

(Contd. on page 2)

Safety data sheet
according to 1907/2006/EC, Article 31

Printing date 04.03.2011 Revision: 04.03.2011

Trade name: Cycloheximide

(Contd. of page 2)

Seek medical attention if symptoms appear.
· **After eye contact:**
Rinse opened eye for several minutes under running water. Then consult a doctor.
· **After ingestion:** Do not induce vomiting; call for medical help immediately.
· **Information for doctor:**
· **Most important symptoms and effects, both acute and delayed**
No further relevant information available.
· **Indication of any immediate medical attention and special treatment needed**
No further relevant information available.

5 Firefighting measures

· **Extinguishing media**
· **Suitable extinguishing agents:**
CO2, powder or water spray. Fight larger fires with water spray or alcohol resistant foam.
· **Special hazards arising from the substance or mixture**
No further relevant information available.
· **Advice for firefighters**
· **Protective equipment:**
Wear self-contained respiratory protective device.
Do not inhale explosion gases or combustion gases.
Wear fully protective suit.

6 Accidental release measures

· **Personal precautions, protective equipment and emergency procedures:**
Avoid formation of dust.
Wear protective clothing.
· **Environmental precautions:**
Inform respective authorities in case of seepage into water course or sewage system.
Do not allow to enter sewers/ surface or ground water.
· **Methods and material for containment and cleaning up:**
Dispose contaminated material as waste according to item 13.
· **Reference to other sections**
See Section 7 for information on safe handling.
See Section 8 for information on personal protection equipment.
See Section 13 for disposal information.

7 Handling and storage

· **Handling:**
· **Precautions for safe handling:**
Thorough dedusting.
Open and handle receptacle with care.
· **Information about fire - and explosion protection:** Keep respiratory protective device available.

· **Conditions for safe storage, including any incompatibilities**
· **Storage:**
· **Requirements to be met by storerooms and receptacles:**
Store tightly sealed in a cool, dry and well ventilated location (see label for storage temperature and additional specific information).
· **Information about storage in one common storage facility:**
Keep away from heat, sources of ignition and incompatibles such as oxidizing agents.
· **Further information about storage conditions:**
Store under lock and key and with access restricted to technical experts or their assistants only.

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Trade name: Cycloheximide

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· **Specific end use(s)** No further relevant information available.

8 Exposure controls/personal protection

· **Additional information about design of technical facilities:** No further data; see item 7.

· **Control parameters**
· **Ingredients with limit values that require monitoring at the workplace:** Not required.
· **Additional information:** The lists valid during the making were used as basis.

· **Exposure controls**
· **Personal protective equipment:**
· **General protective and hygienic measures:**
Keep away from foodstuffs, beverages and feed.
Immediately remove all soiled and contaminated clothing
Wash hands before breaks and at the end of work.
Store protective clothing separately.
· **Respiratory protection:** Not required.
· **Protection of hands:**

Protective gloves

The glove material has to be impermeable and resistant to the product/ the substance/ the preparation.
Due to missing tests no recommendation to the glove material can be given for the product/the preparation/the chemical mixture.
Selection of the glove material on consideration of the penetration times, rates of diffusion and the degradation
· **Material of gloves**
The selection of the suitable gloves does not only depend on the material, but also on further marks of quality and varies from manufacturer to manufacturer.
· **Penetration time of glove material**
The exact break through time has to be found out by the manufacturer of the protective gloves and has to be observed.
· **Eye protection:**

Tightly sealed goggles

9 Physical and chemical properties

· **Information on basic physical and chemical properties**	
· **General Information**	
· **Appearance:**	
Form:	Powder
Colour:	White to yellow
· **Odour:**	Characteristic
· **Odour threshold:**	Not determined.
· **pH-value:**	Not applicable.
· **Change in condition**	
Melting point/Melting range:	116 (Z)°C
Boiling point/Boiling range:	Undetermined.

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Trade name: Cycloheximide

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· **Flash point:**	Not applicable.
· **Flammability (solid, gaseous):**	Product is not flammable.
· **Ignition temperature:**	
Decomposition temperature:	Not determined.
· **Self-igniting:**	Not determined.
· **Danger of explosion:**	Product does not present an explosion hazard.
· **Explosion limits:**	
Lower:	Not determined.
Upper:	Not determined.
· **Vapour pressure:**	Not applicable.
· **Density:**	Not determined.
· **Relative density**	Not determined.
· **Vapour density**	Not applicable.
· **Evaporation rate**	Not applicable.
· **Solubility in / Miscibility with water:**	Not determined.
· **Segregation coefficient (n-octanol/water):**	Not determined.
· **Viscosity:**	
Dynamic:	Not applicable.
Kinematic:	Not applicable.
· **Other information**	No further relevant information available.

10 Stability and reactivity

· **Reactivity**
· **Chemical stability:** Stable. Avoid strong oxidizing agents.
· **Thermal decomposition / conditions to be avoided:**
No decomposition if used according to specifications.
· **Possibility of hazardous reactions:** No dangerous reactions known.
· **Conditions to avoid:** No further relevant information available.
· **Incompatible materials:** No further relevant information available.
· **Hazardous decomposition products:** No dangerous decomposition products known.

11 Toxicological information

· **Information on toxicological effects**
· **Acute toxicity:**

· **LD/LC50 values relevant for classification:**

Oral	LD50	65 mg/kg (dog)
		65 mg/kg (guinea pig)
		60 mg/kg (monkey)
		133 mg/kg (mouse)
		2 mg/kg (rat)
	LD50	4 mg/kg (cat) (intraperitoneal)
		1 mg/kg (dog) (intravenous)
		60 mg/kg (guinea pig) (intraperitoneal)

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		60 mg/kg (guinea pig) (subcutaneous)
		100 mg/kg (mouse) (intraperitoneal)
		150 mg/kg (mouse) (intravenous)
		160 mg/kg (mouse) (subcutaneous)
		3,7 mg/kg (rat) (intraperitoneal)
		2 mg/kg (rat) (intravenous)
		17 mg/kg (rabbit) (intravenous)

· **Primary irritant effect:**
· **on the skin:** Not determined
· **on the eye:** Not determined
· **Sensitization:** No sensitizing effects known.
· **CMR effects (carcinogenity, mutagenicity and toxicity for reproduction)**
Muta. Cat. 3, Repr. Cat. 2

12 Ecological information

· **Toxicity**
· **Aquatic toxicity:** No further relevant information available.
· **Persistence and degradability:** No further relevant information available.
· **Behaviour in environmental systems:**
· **Bioaccumulative potential:** No further relevant information available.
· **Mobility in soil:** No further relevant information available.
· **Ecotoxical effects:**
· **Remark:** Toxic for fish
· **Additional ecological information:**
· **General notes:**
Water hazard class 3 (German Regulation) (Assessment by list): extremely hazardous for water
Do not allow product to reach ground water, water course or sewage system, even in small quantities.
Danger to drinking water if even extremely small quantities leak into the ground.
Also poisonous for fish and plankton in water bodies.
Toxic for aquatic organisms
· **Results of PBT and vPvB assessment**
· **PBT:** Not applicable.
· **vPvB:** Not applicable.
· **Other adverse effects** No further relevant information available.

13 Disposal considerations

· **Waste treatment methods**
· **Recommendation:**
Must not be disposed together with household garbage. Do not allow product to reach sewage system.

· **Uncleaned packaging:**
· **Recommendation:** Disposal must be made according to official regulations.

EU

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Trade name: Cycloheximide

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14 Transport information

· **Land transport ADR/RID (cross-border)**

· **ADR/RID class:**	6.1 Toxic substances.
· **Danger code (Kemler):**	66
· **UN-Number:**	2588
· **Packaging group:**	I
· **Hazard label:**	6.1
· **Special marking:**	Symbol (fish and tree)
· **UN proper shipping name:**	2588 PESTICIDE, SOLID, TOXIC, N.O.S. (Cycloheximide)
· **Tunnel restriction code**	C/E

· **Maritime transport IMDG:**

· **IMDG Class:**	6.1
· **UN Number:**	2588
· **Label**	6.1
· **Packaging group:**	I
· **EMS Number:**	F-A,S-A
· **Marine pollutant:**	No
· **Proper shipping name:**	PESTICIDE, SOLID, TOXIC, N.O.S. (Cycloheximide)

· **Air transport ICAO-TI and IATA-DGR:**

· **ICAO/IATA Class:**	6.1
· **UN/ID Number:**	2588
· **Label**	6.1
· **Packaging group:**	I
· **Proper shipping name:**	PESTICIDE, SOLID, TOXIC, N.O.S. (Cycloheximide)

· **UN "Model Regulation":** UN2588, PESTICIDE, SOLID, TOXIC, N.O.S., 6.1, I
· **Environmental hazards:** Environmentally hazardous substance, solid; Marine Pollutant
· **Special precautions for user:** Warning: Toxic substances.
· **Transport in bulk according to Annex II of MARPOL73/78 and the IBC Code:** Not applicable.

15 Regulatory information

· **Chemical safety assessment:** A Chemical Safety Assessment has not been carried out.

16 Other information

This information is based on our present knowledge. However, this shall not constitute a guarantee for any specific product features and shall not establish a legally valid contractual relationship. For research use only. Not for drug, household or other uses.

· **Department issuing MSDS:** Customer service
· **Contact:** tel. +1 610-941-0430

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Safety data sheet
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Trade name: Cycloheximide

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· **Abbreviations and acronyms:**
ADR: Accord européen sur le transport des marchandises dangereuses par Route (European Agreement concerning the International Carriage of Dangerous Goods by Road)
RID: Réglement international concernant le transport des marchandises dangereuses par chemin de fer (Regulations Concerning the International Transport of Dangerous Goods by Rail)
IMDG: International Maritime Code for Dangerous Goods
IATA: International Air Transport Association
IATA-DGR: Dangerous Goods Regulations by the "International Air Transport Association" (IATA)
ICAO: International Civil Aviation Organization
ICAO-TI: Technical Instructions by the "International Civil Aviation Organization" (ICAO)
GHS: Globally Harmonized System of Classification and Labelling of Chemicals
EINECS: European Inventory of Existing Commercial Chemical Substances
CAS: Chemical Abstracts Service (division of the American Chemical Society)
LC50: Lethal concentration, 50 percent
LD50: Lethal dose, 50 percent

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Trade name: MG-132

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· **Segregation coefficient (n-octanol/water):** Not determined.

· **Viscosity:**
Dynamic: Not applicable.
Kinematic: Not applicable.
· **Other information** No further relevant information available.

10 Stability and reactivity

· **Reactivity**
· **Chemical stability:** Stable. Avoid strong oxidizing agents.
· **Thermal decomposition / conditions to be avoided:**
No decomposition if used according to specifications.
· **Possibility of hazardous reactions:** No dangerous reactions known.
· **Conditions to avoid:** No further relevant information available.
· **Incompatible materials:** No further relevant information available.
· **Hazardous decomposition products:** No dangerous decomposition products known.

11 Toxicological information

· **Information on toxicological effects**
· **Acute toxicity:**
· **Primary irritant effect:**
· **on the skin:** Not determined
· **on the eye:** Not determined
· **Sensitization:** No sensitizing effects known.
· **Additional toxicological information:**
When used and handled according to specifications, the product does not have any harmful effects to our experience and the information provided to us.
The substance is not subject to classification according to the latest version of the EU lists.

12 Ecological information

· **Toxicity**
· **Aquatic toxicity:** No further relevant information available.
· **Persistence and degradability:** No further relevant information available.
· **Behaviour in environmental systems:**
· **Bioaccumulative potential:** No further relevant information available.
· **Mobility in soil:** No further relevant information available.
· **Results of PBT and vPvB assessment**
· **PBT:** Not applicable.
· **vPvB:** Not applicable.
· **Other adverse effects** No further relevant information available.

13 Disposal considerations

· **Waste treatment methods**
· **Recommendation:** Smaller quantities can be disposed of with household waste.

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Trade name: MG-132

(Contd. of page 1)

· **Information for doctor:**
· **Most important symptoms and effects, both acute and delayed**
No further relevant information available.
· **Indication of any immediate medical attention and special treatment needed**
No further relevant information available.

5 Firefighting measures

· **Extinguishing media**
· **Suitable extinguishing agents:**
CO2, powder or water spray. Fight larger fires with water spray or alcohol resistant foam.
· **Special hazards arising from the substance or mixture** No further relevant information available.
· **Advice for firefighters**
· **Protective equipment:**
Wear self-contained respiratory protective device.
Do not inhale explosion gases or combustion gases.
Wear fully protective suit.

6 Accidental release measures

· **Personal precautions, protective equipment and emergency procedures:**
Avoid formation of dust.
Wear protective clothing.
· **Environmental precautions:** No special measures required.
· **Methods and material for containment and cleaning up:** Pick up mechanically.
· **Reference to other sections**
No dangerous substances are released.
See Section 7 for information on safe handling.
See Section 8 for information on personal protection equipment.
See Section 13 for disposal information.

7 Handling and storage

· **Handling:**
· **Precautions for safe handling:** No special measures required.
· **Information about fire - and explosion protection:** No special measures required.

· **Conditions for safe storage, including any incompatibilities**
· **Storage:**
· **Requirements to be met by storerooms and receptacles:**
Store tightly sealed in a cool, dry and well ventilated location (see label for storage temperature and additional specific information).
· **Information about storage in one common storage facility:**
Keep away from heat, sources of ignition and incompatibles such as oxidizing agents.
· **Further information about storage conditions:**
Store under lock and key and with access restricted to technical experts or their assistants only.
· **Specific end use(s)** No further relevant information available.

8 Exposure controls/personal protection

· **Additional information about design of technical facilities:** No further data; see item 7.

· **Control parameters**
· **Ingredients with limit values that require monitoring at the workplace:** Not required.
· **Additional information:** The lists valid during the making were used as basis.

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1 Identification of the substance/mixture and of the company/undertaking

· **Product identifier**

· **Trade name:** MG-132
· **Synonyms:** Z-Leu-Leu-Leu-CHO

· **Article number:** BML-PI102
· **CAS Number:**
133407-82-6
· **Relevant identified uses of the substance or mixture and uses advised against**
· **Application of the substance / the preparation:** Laboratory chemicals

· **Details of the supplier of the safety data sheet**
· **Manufacturer/Supplier:**
Enzo Life Sciences International, Inc.
10 Executive Boulevard
Farmingdale, NY 11735
U.S.A.
msds@enzolifesciences.com

· **Further information obtainable from:** Customer service
· **Emergency telephone number:** During normal opening times: +1 610-941-0430

2 Hazards identification

· **Classification of the substance or mixture**
· **Classification according to Regulation (EC) No 1272/2008**
The substance is not classified according to the CLP regulation.

· **Classification according to Directive 67/548/EEC or Directive 1999/45/EC:** Not applicable.
· **Information concerning particular hazards for human and environment:** Not applicable.

· **Label elements**
· **Labelling according to Regulation (EC) No 1272/2008:** Void
· **Hazard pictograms:** Void
· **Signal word:** Void
· **Hazard statements:** Void
· **Other hazards**
· **Results of PBT and vPvB assessment**
· **PBT:** Not applicable.
· **vPvB:** Not applicable.

3 Composition/information on ingredients

· **Chemical characterization: Substances**
· **CAS No. Description:**
133407-82-6 MG-132
· **Identification number(s):** Not applicable

4 First aid measures

· **Description of first aid measures**
· **General information:** No special measures required.
· **After inhalation:** Supply fresh air; consult doctor in case of complaints.
· **After skin contact:** Remove contaminated clothing.
· **After eye contact:** Rinse opened eye for several minutes under running water.
· **After ingestion:** If symptoms persist consult doctor.

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Trade name: MG-132

(Contd. of page 2)

· **Exposure controls**
· **Personal protective equipment:**
· **General protective and hygienic measures:**
The usual precautionary measures are to be adhered to when handling chemicals.
· **Respiratory protection:** Not required.
· **Protection of hands:**
The glove material has to be impermeable and resistant to the product/ the substance/ the preparation.
Due to missing tests no recommendation to the glove material can be given for the product/the preparation/the chemical mixture.
Selection of the glove material on consideration of the penetration times, rates of diffusion and the degradation
· **Material of gloves**
The selection of the suitable gloves does not only depend on the material, but also on further marks of quality and varies from manufacturer to manufacturer.
· **Penetration time of glove material**
The exact break through time has to be found out by the manufacturer of the protective gloves and has to be observed.
· **Eye protection:** Not required.

9 Physical and chemical properties

· **Information on basic physical and chemical properties**	
· **General Information**	
· **Appearance:**	
Form:	Solid
Colour:	White
· **Odour:**	Characteristic
· **Odour threshold:**	Not determined.
· **pH-value:**	Not applicable.
· **Change in condition**	
Melting point/Melting range:	Undetermined.
Boiling point/Boiling range:	Undetermined.
· **Flash point:**	Not applicable.
· **Flammability (solid, gaseous):**	Product is not flammable.
· **Ignition temperature:**	
Decomposition temperature:	Not determined.
· **Self-igniting:**	Not determined.
· **Danger of explosion:**	Product does not present an explosion hazard.
· **Explosion limits:**	
Lower:	Not determined.
Upper:	Not determined.
· **Vapour pressure:**	Not applicable.
· **Density:**	Not determined.
· **Relative density**	Not determined.
· **Vapour density**	Not applicable.
· **Evaporation rate**	Not applicable.
· **Solubility in / Miscibility with water:**	Insoluble.

(Contd. on page 4)

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Trade name: MG-132

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· **Uncleaned packaging:**
· **Recommendation:** Disposal must be made according to official regulations.

14 Transport information

· **UN-Number**	Not applicable
· **UN proper shipping name**	Not applicable
· **Transport hazard class(es)**	Not applicable
· **Packing group**	Not applicable
· **Environmental hazards:** · **Marine pollutant:**	No
· **Special precautions for user:**	Not applicable.
· **Transport in bulk according to Annex II of MARPOL73/78 and the IBC Code:**	Not applicable.

15 Regulatory information

· **Chemical safety assessment:** A Chemical Safety Assessment has not been carried out.

16 Other information

This information is based on our present knowledge. However, this shall not constitute a guarantee for any specific product features and shall not establish a legally valid contractual relationship. For research use only. Not for drug, household or other uses.

· **Department issuing MSDS:** Customer service
· **Contact:** tel. +1 610-941-0430
· **Abbreviations and acronyms:**
GHS: Globally Harmonized System of Classification and Labelling of Chemicals
CAS: Chemical Abstracts Service (division of the American Chemical Society)

SIGMA-ALDRICH

sigma-aldrich.com

Material Safety Data Sheet

Version 3.1
Revision Date 10/23/2010
Print Date 08/29/2011

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : Calpain Inhibitor I

Product Number : A6185
Brand : Sigma
Product Use : For laboratory research purposes.

Supplier : Sigma-Aldrich Canada, Ltd
2149 Winston Park Drive
OAKVILLE ON L6H 6J8
CANADA

Manufacturer : Sigma-Aldrich Corporation
3050 Spruce St.
St. Louis, Missouri 63103
USA

Telephone : +1 9058299500
Fax : +1 9058299292
Emergency Phone # (For both supplier and manufacturer) : 1-800-424-9300
Preparation Information : Sigma-Aldrich Corporation
Product Safety - Americas Region
1-800-521-8956

2. HAZARDS IDENTIFICATION

Emergency Overview

WHMIS Classification

Not WHMIS controlled.

Not WHMIS controlled.

Not a dangerous substance according to GHS.

HMIS Classification

Health hazard: 0
Flammability: 0
Physical hazards: 0

Potential Health Effects

Inhalation May be harmful if inhaled. May cause respiratory tract irritation.
Skin May be harmful if absorbed through skin. May cause skin irritation.
Eyes May cause eye irritation.
Ingestion May be harmful if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : ALLN
MG-101
Ac-LLnL-CHO
N-Acetyl-Leu-Leu-Norleu-al
N-Acetyl-L-leucyl-L-leucyl-L-norleucinal

Formula : $C_{20}H_{37}N_3O_4$
Molecular Weight : 383.53 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
Calpain Inhibitor I			
110044-82-1	-	-	-

4. FIRST AID MEASURES

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration.

In case of skin contact
Wash off with soap and plenty of water.

In case of eye contact
Flush eyes with water as a precaution.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water.

5. FIRE-FIGHTING MEASURES

Conditions of flammability
Not flammable or combustible.

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)

Explosion data - sensitivity to mechanical impact
no data available

Explosion data - sensitivity to static discharge
no data available

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Avoid dust formation. Avoid breathing vapors, mist or gas.

Environmental precautions
Do not let product enter drains.

Methods and materials for containment and cleaning up
Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: -20 °C

Keep in a dry place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
Respiratory protection is not required. Where protection from nuisance levels of dusts are desired, use type N95 (US) or type P1 (EN 143) dust masks. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
Choose body protection in relation to its type, to the concentration and amount of dangerous substances, and to the specific work-place., The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
General industrial hygiene practice.

Specific engineering controls
Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	powder
Colour	white

Safety data

pH	no data available
Melting/freezing point	no data available
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available
Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
no data available

Inhalation LC50
no data available

Dermal LD50
no data available

Other information on acute toxicity
no data available

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization
no data available

Germ cell mutagenicity
no data available

Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
no data available

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. May cause respiratory tract irritation.
Ingestion	May be harmful if swallowed.
Skin	May be harmful if absorbed through skin. May cause skin irritation.
Eyes	May cause eye irritation.

Signs and Symptoms of Exposure
To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Synergistic effects
no data available

Additional Information
RTECS: Not available

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

DSL Status
This product contains the following components that are not on the Canadian DSL nor NDSL lists.

	CAS-No.
Calpain Inhibitor I	110044-82-1

WHMIS Classification

Not WHMIS controlled. Not WHMIS controlled.

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Safety data sheet
according to 1907/2006/EC, Article 31

Printing date 08.12.2010 Revision: 08.12.2010

Trade name: Y-27632 hydrochloride

(Contd. of page 2)

6 Accidental release measures

· **Personal precautions, protective equipment and emergency procedures:**
Avoid formation of dust.
Wear protective clothing.
· **Environmental precautions:** No special measures required.
· **Methods and material for containment and cleaning up:**
Dispose contaminated material as waste according to item 13.
Ensure adequate ventilation.
· **Reference to other sections**
See Section 7 for information on safe handling.
See Section 8 for information on personal protection equipment.
See Section 13 for disposal information.

7 Handling and storage

· **Handling:**
· **Precautions for safe handling:**
Prevent formation of dust.
Ensure good ventilation/exhaustion at the workplace.
· **Information about fire - and explosion protection:** No special measures required.

· **Conditions for safe storage, including any incompatibilities**
· **Storage:**
· **Requirements to be met by storerooms and receptacles:**
Store tightly sealed in a cool, dry and well ventilated location (see label for storage temperature and additional specific information).
· **Information about storage in one common storage facility:**
Keep away from heat, sources of ignition and incompatibles such as oxidizing agents.
· **Further information about storage conditions:**
Keep container tightly sealed.
Store under lock and key and with access restricted to technical experts or their assistants only.
· **Specific end use(s)** No further relevant information available.

8 Exposure controls/personal protection

· **Additional information about design of technical facilities:** No further data; see item 7.

· **Control parameters**
· **Ingredients with limit values that require monitoring at the workplace:** Not required.
· **Additional information:** The lists valid during the making were used as basis.

· **Exposure controls**
· **Personal protective equipment:**
· **General protective and hygienic measures:**
Keep away from foodstuffs, beverages and feed.
Wash hands before breaks and at the end of work.
Avoid contact with the eyes and skin.
· **Respiratory protection:**
In case of brief exposure or low pollution use respiratory filter device. In case of intensive or longer exposure use self-contained respiratory protective device.
· **Protection of hands:**

Protective gloves

(Contd. on page 4)

Safety data sheet
according to 1907/2006/EC, Article 31

Printing date 08.12.2010 Revision: 08.12.2010

1 Identification of the substance/mixture and of the company/undertaking

· **Product identifier**

· **Trade name:** Y-27632 hydrochloride
· **Synonyms:** (R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide . 2HCl

· **Article number:** ALX-270-333
· **CAS Number:**
146986-50-7
· **Relevant identified uses of the substance or mixture and uses advised against**
· **Application of the substance / the preparation:** Laboratory chemicals

· **Details of the supplier of the safety data sheet**
· **Manufacturer/Supplier:**
Enzo Life Sciences International, Inc.
10 Executive Boulevard
Farmingdale, NY 11735
U.S.A.
msds@enzolifesciences.com

· **Further information obtainable from:** Customer service
· **Emergency telephone number:** During normal opening times: +1 610-941-0430

2 Hazards identification

· **Classification of the substance or mixture**
· **Classification according to Regulation (EC) No 1272/2008**


GHS07

Acute Tox. 4 H302 Harmful if swallowed.
Acute Tox. 4 H312 Harmful in contact with skin.
Acute Tox. 4 H332 Harmful if inhaled.

· **Classification according to Directive 67/548/EEC or Directive 1999/45/EC:**


Xn; Harmful

R20/21/22-48: Harmful by inhalation, in contact with skin and if swallowed. Danger of serious damage to health by prolonged exposure.

· **Information concerning particular hazards for human and environment:** Not applicable.

· **Label elements**
· **Labelling according to Regulation (EC) No 1272/2008:**
The substance is classified and labelled according to the CLP regulation.
· **Hazard pictograms:**


GHS07

· **Signal word:** Warning

· **Hazard-determining components of labelling:** Void
· **Hazard statements:**
H302 Harmful if swallowed.
H312 Harmful in contact with skin.
H332 Harmful if inhaled.
· **Precautionary statements**
P261 Avoid breathing dust/fume/gas/mist/vapours/spray.
P280 Wear protective gloves/protective clothing/eye protection/face protection.

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Safety data sheet
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Trade name: Y-27632 hydrochloride

(Contd. of page 3)

The glove material has to be impermeable and resistant to the product/ the substance/ the preparation.
Due to missing tests no recommendation to the glove material can be given for the product/the preparation/the chemical mixture.
Selection of the glove material on consideration of the penetration times, rates of diffusion and the degradation

· **Material of gloves**
The selection of the suitable gloves does not only depend on the material, but also on further marks of quality and varies from manufacturer to manufacturer.

· **Penetration time of glove material**
The exact break through time has to be found out by the manufacturer of the protective gloves and has to be observed.

· **Eye protection:** Not required.

9 Physical and chemical properties

· **Information on basic physical and chemical properties**	
· **General Information**	
· **Appearance:**	
Form:	Not determined.
Colour:	Not determined.
· **Odour:**	Characteristic
· **Odour threshold:**	Not determined.
· **pH-value:**	Not determined.
· **Change in condition**	
Melting point/Melting range:	Undetermined.
Boiling point/Boiling range:	Undetermined.
· **Flash point:**	Not applicable.
· **Ignition temperature:**	
Decomposition temperature:	Not determined.
· **Self-igniting:**	Not determined.
· **Danger of explosion:**	Product does not present an explosion hazard.
· **Explosion limits:**	
Lower:	Not determined.
Upper:	Not determined.
· **Vapour pressure:**	Not determined.
· **Density:**	Not determined.
· **Relative density**	Not determined.
· **Vapour density**	Not determined.
· **Evaporation rate**	Not applicable.
· **Solubility in / Miscibility with water:**	Not determined.
· **Segregation coefficient (n-octanol/water):**	Not determined.
· **Viscosity:**	
Dynamic:	Not determined.
Kinematic:	Not determined.
· **Other information**	No further relevant information available.

EU

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Trade name: Y-27632 hydrochloride

(Contd. of page 1)

P322 Specific measures (see on this label).
P301+P312 IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell.
P304+P340 IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.
P501 Dispose of contents/container in accordance with local/regional/national/international regulations.

· **Other hazards**
· **Results of PBT and vPvB assessment**
· **PBT:** Not applicable.
· **vPvB:** Not applicable.

3 Composition/information on ingredients

· **Chemical characterization: Substances**
· **CAS No. Description:**
146986-50-7 Y-27632 hydrochloride
· **Identification number(s):** Not determined

4 First aid measures

· **Description of first aid measures**
· **General information:**
Symptoms of poisoning may even occur after several hours; therefore medical observation for at least 48 hours after the accident.
· **After inhalation:**
Supply fresh air. If required, provide artificial respiration. Keep patient warm. Consult doctor if symptoms persist.
In case of unconsciousness place patient stably in side position for transportation.
· **After skin contact:**
Immediately wash with water and soap and rinse thoroughly.
Remove contaminated clothing.
· **After eye contact:**
Rinse opened eye for several minutes under running water. Check for and remove contact lenses. If symptoms persist, consult a doctor.
· **After ingestion:** Call for a doctor immediately.
· **Information for doctor:**
· **Most important symptoms and effects, both acute and delayed**
No further relevant information available.
· **Indication of any immediate medical attention and special treatment needed**
No further relevant information available.

5 Firefighting measures

· **Extinguishing media**
· **Suitable extinguishing agents:**
CO2, powder or water spray. Fight larger fires with water spray or alcohol resistant foam.
· **Special hazards arising from the substance or mixture** No further relevant information available.
· **Advice for firefighters**
· **Protective equipment:**
Mouth respiratory protective device.
Wear self-contained respiratory protective device.
Do not inhale explosion gases or combustion gases.
Wear fully protective suit.

(Contd. on page 3)

Safety data sheet
according to 1907/2006/EC, Article 31

Printing date 08.12.2010 Revision: 08.12.2010

Trade name: Y-27632 hydrochloride

(Contd. of page 4)

10 Stability and reactivity

· **Reactivity**
· **Chemical stability:** Stable. Avoid strong oxidizing agents.
· **Thermal decomposition / conditions to be avoided:**
No decomposition if used according to specifications.
· **Possibility of hazardous reactions:** No dangerous reactions known.
· **Conditions to avoid:** No further relevant information available.
· **Incompatible materials:** No further relevant information available.
· **Hazardous decomposition products:** No dangerous decomposition products known.

11 Toxicological information

· **Information on toxicological effects**
· **Acute toxicity:**
· **Primary irritant effect:**
· **on the skin:** No irritant effect.
· **on the eye:** No irritating effect.
· **Sensitization:** No sensitizing effects known.

12 Ecological information

· **Toxicity**
· **Aquatic toxicity:** No further relevant information available.
· **Persistence and degradability:** No further relevant information available.
· **Behaviour in environmental systems:**
· **Bioaccumulative potential:** No further relevant information available.
· **Mobility in soil:** No further relevant information available.
· **Results of PBT and vPvB assessment**
· **PBT:** Not applicable.
· **vPvB:** Not applicable.
· **Other adverse effects** No further relevant information available.

13 Disposal considerations

· **Waste treatment methods**
· **Recommendation:**
Must not be disposed together with household garbage. Do not allow product to reach sewage system.

· **Uncleaned packaging:**
· **Recommendation:** Disposal must be made according to official regulations.

14 Transport information

· **Maritime transport IMDG:**
· **Marine pollutant:** No
· **Special precautions for user:** Not applicable.
· **Transport in bulk according to Annex II of MARPOL73/78 and the IBC Code:** Not applicable.

EU

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Safety data sheet
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Trade name: Y-27632 hydrochloride

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15 Regulatory information

· **Chemical safety assessment:** A Chemical Safety Assessment has not been carried out.

16 Other information

This information is based on our present knowledge. However, this shall not constitute a guarantee for any specific product features and shall not establish a legally valid contractual relationship. For research use only. Not for drug, household or other uses.

· **Department issuing MSDS:** Customer service
· **Contact:** tel. +1 610-941-0430
· **Abbreviations and acronyms:**
IMDG: International Maritime Code for Dangerous Goods
GHS: Globally Harmonized System of Classification and Labelling of Chemicals
CAS: Chemical Abstracts Service (division of the American Chemical Society)

BIO BASIC INC.

160 Torbay Road, Markham Ontario. L3R 1G6 Canada
Tel: (905) 474 4493, (800) 313 7224 Fax: (905) 474 5794

MATERIAL SAFETY DATA SHEET
REQUIRED UNDER SAFETY AND HEALTH REGULATION FOR SHIP REPAIRING

MANUFACTURER'S NAME:	BIO BASIC INC.
TEL:	(905) 474 5794
FAX:	(416) 995 9730
FOR CHEMICAL EMERGENCY:	(416) 995 9730
ADDRESS:	160 Torbay Road, Markham Ontario. L3R 1G6 Canada

UPDATED: May 2007

SECTION 1. - - - - - - - - - CHEMICAL IDENTIFICATION- - - - - - - - - -
CODE#: DB0058
NAME: DTT (DL-DITHIOTHREITOL)
SECTION 2. - - - - - COMPOSITION/INFORMATION ON INGREDIENTS - - - - - -
CHEMICAL NAME: CAS#: RTECS #: %:
DL-DITHIOTHREITOL 3483-12-3 XO8576500 >98.5
FOR MORE INFORMATION SEE COMPLETE RTECS ENTRY.
MF: C4H10O2S2
EC NO: 248-531-9
SYNONYMS
2,3-BUTANEDIOL, 1,4-DIMERCAPTO-, DL-THREO- * 2,3-BUTANEDIOL, 1,4-DIMERCAPTO-, (R*,R*)- (+-)- (9CI) * CLELAND'S REAGENT * DL-THREO-1,4-DIMERCAPTO-2,3-BUTANEDIOL * (R*,R*)-(+-)-1,4-DIMERCAPTO-2,3-BUTANEDIOL * DITHIOTHREITOL * DL-DITHIOTHREITOL * DL-1,4-DITHIOTHREITOL * RAC-DITHIOTHREITOL *
SECTION 3. - - - - - - - - - - HAZARDS IDENTIFICATION - - - - - - - - -
LABEL PRECAUTIONARY STATEMENTS
IRRITANT
IRRITATING TO EYES, RESPIRATORY SYSTEM AND SKIN.
TOXIC
HARMFUL BY INHALATION, IN CONTACT WITH SKIN AND IF SWALLOWED.
TARGET ORGAN(S):
CENTRAL NERVOUS SYSTEM
IN CASE OF CONTACT WITH EYES, RINSE IMMEDIATELY WITH PLENTY OF WATER AND SEEK MEDICAL ADVICE.
WEAR SUITABLE PROTECTIVE CLOTHING.
SECTION 4. - - - - - - - - - - FIRST-AID MEASURES- - - - - - - - - - -
IN CASE OF CONTACT, IMMEDIATELY WASH SKIN WITH SOAP AND COPIOUS AMOUNTS OF WATER.
IN CASE OF CONTACT, IMMEDIATELY FLUSH EYES WITH COPIOUS AMOUNTS OF WATER FOR AT LEAST 15 MINUTES.
IF INHALED, REMOVE TO FRESH AIR. IF NOT BREATHING GIVE ARTIFICIAL RESPIRATION. IF BREATHING IS DIFFICULT, GIVE OXYGEN.
IF SWALLOWED, WASH OUT MOUTH WITH WATER PROVIDED PERSON IS CONSCIOUS. CALL A PHYSICIAN.
DISCARD CONTAMINATED CLOTHING AND SHOES.
SECTION 5. - - - - - - - - - FIRE FIGHTING MEASURES - - - - - - - - - -
EXTINGUISHING MEDIA
CARBON DIOXIDE, DRY CHEMICAL POWDER OR APPROPRIATE FOAM.

SPECIAL FIREFIGHTING PROCEDURES

EXPOSURE CAN CAUSE:
NAUSEA, HEADACHE AND VOMITING
CNS DEPRESSION
CHRONIC EFFECTS
TARGET ORGAN(S):
CENTRAL NERVOUS SYSTEM
TO THE BEST OF OUR KNOWLEDGE, THE CHEMICAL, PHYSICAL, AND
TOXICOLOGICAL PROPERTIES HAVE NOT BEEN THOROUGHLY INVESTIGATED.
RTECS #: XO8576500
THREITOL, 1,4-DITHIO-, DL-
TOXICITY DATA
ORL-RAT LD50: 400 MG/KG
IPR-MUS LD50:169 MG/KG JMCMAR 15,600,1972
SCU-MUS LD50:333 MG/KG ARZNAD 22,1434,1972
IVN-MUS LD50:94 MG/KG ARZNAD 22,1434,1972
ONLY SELECTED REGISTRY OF TOXIC EFFECTS OF CHEMICAL SUBSTANCES
(RTECS) DATA IS PRESENTED HERE. SEE ACTUAL ENTRY IN RTECS FOR
COMPLETE INFORMATION.
SECTION 12. - - - - - - - - - ECOLOGICAL INFORMATION - - - - - - - - - -
DATA NOT YET AVAILABLE.
SECTION 13. - - - - - - - - - DISPOSAL CONSIDERATIONS - - - - - - - - -
OBSERVE ALL FEDERAL, STATE AND LOCAL ENVIRONMENTAL REGULATIONS.
SECTION 14. - - - - - - - - - - TRANSPORT INFORMATION - - - - - - - - -
NOT REGULATED.
SECTION 15. - - - - - - - - - REGULATORY INFORMATION - - - - - - - - - -
EUROPEAN INFORMATION
TOXIC
R 23/24/25
TOXIC BY INHALATION, IN CONTACT WITH SKIN AND IF SWALLOWED.
IRRITANT
R 36/37/38
IRRITATING TO EYES, RESPIRATORY SYSTEM AND SKIN.
S: 36/37/39
WEAR SUITABLE PROTECTIVE CLOTHING, GLOVES AND EYE/FACE PROTECTION.
REVIEWS, STANDARDS, AND REGULATIONS
OEL=MAK
NOES 1983: HZD X2950; NIS 1; TNF 35; NOS 3; TNE 627; TFE 436
SECTION 16. - - - - - - - - - - OTHER INFORMATION- - - - - - - - - - -
THE ABOVE INFORMATION IS BELIEVED TO BE CORRECT BUT DOES NOT PURPORT TO
BE ALL INCLUSIVE AND SHALL BE USED ONLY AS A GUIDE. BIO BASIC INC. SHALL
NOT
BE HELD LIABLE FOR ANY DAMAGE RESULTING FROM HANDLING OR FROM CONTACT
WITH THE ABOVE PRODUCT. SEE REVERSE SIDE OF INVOICE OR PACKING SLIP FOR
ADDITIONAL TERMS AND CONDITIONS OF SALE.

WEAR SELF-CONTAINED BREATHING APPARATUS AND PROTECTIVE CLOTHING TO PREVENT CONTACT WITH SKIN AND EYES.
USE WATER SPRAY TO COOL FIRE-EXPOSED CONTAINERS.
UNUSUAL FIRE AND EXPLOSIONS HAZARDS
EMITS TOXIC FUMES UNDER FIRE CONDITIONS.
SECTION 6. - - - - - - - - ACCIDENTAL RELEASE MEASURES- - - - - - - - -
EVACUATE AREA.
WEAR SELF-CONTAINED BREATHING APPARATUS, RUBBER BOOTS AND HEAVY RUBBER GLOVES.
SWEEP UP, PLACE IN A BAG AND HOLD FOR WASTE DISPOSAL.
AVOID RAISING DUST.
VENTILATE AREA AND WASH SPILL SITE AFTER MATERIAL PICKUP IS COMPLETE.
SECTION 7. - - - - - - - - - - HANDLING AND STORAGE- - - - - - - - - -
REFER TO SECTION 8.
SECTION 8. - - - - - - EXPOSURE CONTROLS/PERSONAL PROTECTION- - - - - -
WEAR APPROPRIATE NIOSH/MSHA-APPROVED RESPIRATOR, CHEMICAL-RESISTANT GLOVES, SAFETY GOGGLES, OTHER PROTECTIVE CLOTHING.
USE ONLY IN A CHEMICAL FUME HOOD.
SAFETY SHOWER AND EYE BATH.
DO NOT BREATHE DUST.
DO NOT GET IN EYES, ON SKIN, ON CLOTHING.
AVOID PROLONGED OR REPEATED EXPOSURE.
WASH THOROUGHLY AFTER HANDLING.
KEEP TIGHTLY CLOSED.
STORE IN A COOL DRY PLACE.
SECTION 9. - - - - - - - PHYSICAL AND CHEMICAL PROPERTIES - - - - - - -
APPEARANCE AND ODOR
SOLID.
UNPLEASANT ODOR.
PHYSICAL PROPERTIES
MELTING POINT: 42 C TO 44 C
FLASHPOINT >230F
>110C
SECTION 10. - - - - - - - - -STABILITY AND REACTIVITY - - - - - - - - -
STABILITY
STABLE.
INCOMPATIBILITIES
BASES
OXIDIZING AGENTS
REDUCING AGENTS
ALKALI METALS
MAY DECOMPOSE ON EXPOSURE TO MOIST AIR OR WATER.
HAZARDOUS COMBUSTION OR DECOMPOSITION PRODUCTS
TOXIC FUMES OF:
CARBON MONOXIDE, CARBON DIOXIDE
SULFUR OXIDES
HYDROGEN SULFIDE GAS
HAZARDOUS POLYMERIZATION
WILL NOT OCCUR.
SECTION 11. - - - - - - - - - TOXICOLOGICAL INFORMATION - - - - - - - -
ACUTE EFFECTS
HARMFUL IF SWALLOWED, INHALED, OR ABSORBED THROUGH SKIN.
CAUSES EYE AND SKIN IRRITATION.
MATERIAL IS IRRITATING TO MUCOUS MEMBRANES AND UPPER

RESPIRATORY TRACT.

Safety data sheet
according to 1907/2006/EC, Article 31

Printing date 03.02.2011 Revision: 03.02.2011

1 Identification of the substance/mixture and of the company/undertaking

· **Product identifier**

· **Trade name:** Bafilomycin A1

· **Article number:** BML-CM110
· **CAS Number:**
88899-55-2
· **Relevant identified uses of the substance or mixture and uses advised against**
· **Application of the substance / the preparation:** Laboratory chemicals

· **Details of the supplier of the safety data sheet**
· **Manufacturer/Supplier:**
Enzo Life Sciences International, Inc.
10 Executive Boulevard
Farmingdale, NY 11735
U.S.A.
msds@enzolifesciences.com

· **Further information obtainable from:** Customer service
· **Emergency telephone number:** During normal opening times: +1 610-941-0430

2 Hazards identification

· **Classification of the substance or mixture**
· **Classification according to Regulation (EC) No 1272/2008**


GHS07

Skin Irrit. 2 H315 Causes skin irritation.
Eye Irrit. 2 H319 Causes serious eye irritation.
STOT SE 3 H335 May cause respiratory irritation.

· **Classification according to Directive 67/548/EEC or Directive 1999/45/EC:**
Xi; Irritant
R36/37/38: Irritating to eyes, respiratory system and skin.
· **Information concerning particular hazards for human and environment:** Not applicable.

· **Label elements**
· **Labelling according to Regulation (EC) No 1272/2008:**
The substance is classified and labelled according to the CLP regulation.
· **Hazard pictograms:**


GHS07

· **Signal word:** Warning
· **Hazard statements:**
H315 Causes skin irritation.
H319 Causes serious eye irritation.
H335 May cause respiratory irritation.
· **Precautionary statements**
P261 Avoid breathing dust/fume/gas/mist/vapours/spray.
P280 Wear protective gloves/protective clothing/eye protection/face protection.
P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P321 Specific treatment (see on this label).

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Trade name: Bafilomycin A1

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P405 Store locked up.
P501 Dispose of contents/container in accordance with local/regional/national/international regulations.

· **Other hazards**
· **Results of PBT and vPvB assessment**
· **PBT:** Not applicable.
· **vPvB:** Not applicable.

3 Composition/information on ingredients

· **Chemical characterization: Substances**
· **CAS No. Description:**
88899-55-2 Bafilomycin A1
· **Identification number(s):** Not applicable

4 First aid measures

· **Description of first aid measures**
· **After inhalation:** In case of unconsciousness place patient stably in side position for transportation.
· **After skin contact:**
Immediately wash with water and soap and rinse thoroughly.
Remove contaminated clothing.
· **After eye contact:**
Rinse opened eye for several minutes under running water. Check for and remove contact lenses. If symptoms persist, consult a doctor.
· **After ingestion:** If symptoms persist consult doctor.
· **Information for doctor:**
· **Most important symptoms and effects, both acute and delayed**
No further relevant information available.
· **Indication of any immediate medical attention and special treatment needed**
No further relevant information available.

5 Firefighting measures

· **Extinguishing media**
· **Suitable extinguishing agents:**
CO2, powder or water spray. Fight larger fires with water spray or alcohol resistant foam.
· **Special hazards arising from the substance or mixture** No further relevant information available.
· **Advice for firefighters**
· **Protective equipment:**
Wear self-contained respiratory protective device.
Do not inhale explosion gases or combustion gases.
Wear fully protective suit.

6 Accidental release measures

· **Personal precautions, protective equipment and emergency procedures:**
Wear protective clothing.
· **Environmental precautions:** No special measures required.
· **Methods and material for containment and cleaning up:**
Absorb with liquid-binding material (sand, diatomite, acid binders, universal binders, sawdust).
Ensure adequate ventilation.
· **Reference to other sections**
See Section 7 for information on safe handling.
See Section 8 for information on personal protection equipment.

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Trade name: Bafilomycin A1

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· **Conditions to avoid:** No further relevant information available.
· **Incompatible materials:** No further relevant information available.
· **Hazardous decomposition products:** No dangerous decomposition products known.

11 Toxicological information

· **Information on toxicological effects**
· **Acute toxicity:**
· **Primary irritant effect:**
· **on the skin:** Irritant to skin and mucous membranes.
· **on the eye:** Irritating effect.
· **Sensitization:** No sensitizing effects known.

12 Ecological information

· **Toxicity**
· **Aquatic toxicity:** No further relevant information available.
· **Persistence and degradability:** No further relevant information available.
· **Behaviour in environmental systems:**
· **Bioaccumulative potential:** No further relevant information available.
· **Mobility in soil:** No further relevant information available.
· **Results of PBT and vPvB assessment**
· **PBT:** Not applicable.
· **vPvB:** Not applicable.
· **Other adverse effects** No further relevant information available.

13 Disposal considerations

· **Waste treatment methods**
· **Recommendation:**
Must not be disposed together with household garbage. Do not allow product to reach sewage system.

· **Uncleaned packaging:**
· **Recommendation:** Disposal must be made according to official regulations.

14 Transport information

· **Maritime transport IMDG:**
· **Marine pollutant:** No
· **Special precautions for user:** Not applicable.
· **Transport in bulk according to Annex II of MARPOL73/78 and the IBC Code:** Not applicable.

15 Regulatory information

· **Chemical safety assessment:** A Chemical Safety Assessment has not been carried out.

16 Other information

This information is based on our present knowledge. However, this shall not constitute a guarantee for any specific product features and shall not establish a legally valid contractual relationship. For research use only. Not for drug, household or other uses.

· **Department issuing MSDS:** Customer service

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Trade name: Bafilomycin A1

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See Section 13 for disposal information.

7 Handling and storage

· **Handling:**
· **Precautions for safe handling:**
Ensure good ventilation/exhaustion at the workplace.
Prevent formation of aerosols.
· **Information about fire - and explosion protection:** No special measures required.

· **Conditions for safe storage, including any incompatibilities**
· **Storage:**
· **Requirements to be met by storerooms and receptacles:**
Store tightly sealed in a cool, dry and well ventilated location (see label for storage temperature and additional specific information).
· **Information about storage in one common storage facility:**
Keep away from heat, sources of ignition and incompatibles such as oxidizing agents.
· **Further information about storage conditions:**
Keep container tightly sealed.
Store under lock and key and with access restricted to technical experts or their assistants only.
· **Specific end use(s)** No further relevant information available.

8 Exposure controls/personal protection

· **Additional information about design of technical facilities:** No further data; see item 7.

· **Control parameters**
· **Ingredients with limit values that require monitoring at the workplace:** Not required.
· **Additional information:** The lists valid during the making were used as basis.

· **Exposure controls**
· **Personal protective equipment:**
· **General protective and hygienic measures:**
Keep away from foodstuffs, beverages and feed.
Immediately remove all soiled and contaminated clothing
Wash hands before breaks and at the end of work.
Avoid contact with the eyes and skin.
· **Respiratory protection:**
In case of brief exposure or low pollution use respiratory filter device. In case of intensive or longer exposure use self-contained respiratory protective device.
· **Protection of hands:**

Protective gloves

The glove material has to be impermeable and resistant to the product/ the substance/ the preparation.
Due to missing tests no recommendation to the glove material can be given for the product/the preparation/the chemical mixture.
Selection of the glove material on consideration of the penetration times, rates of diffusion and the degradation
· **Material of gloves**
The selection of the suitable gloves does not only depend on the material, but also on further marks of quality and varies from manufacturer to manufacturer.
· **Penetration time of glove material**
The exact break through time has to be found out by the manufacturer of the protective gloves and has to be observed.

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Trade name: Bafilomycin A1

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· **Eye protection:**

Tightly sealed goggles

9 Physical and chemical properties

· **Information on basic physical and chemical properties**	
· **General Information**	
· **Appearance:**	
Form:	Oily
Colour:	Colourless
· **Odour:**	Characteristic
· **Odour threshold:**	Not determined.
· **pH-value:**	Not determined.
· **Change in condition**	
Melting point/Melting range:	Undetermined.
Boiling point/Boiling range:	Undetermined.
· **Flash point:**	Not applicable.
· **Flammability (solid, gaseous):**	Not applicable.
· **Ignition temperature:**	
Decomposition temperature:	Not determined.
· **Self-igniting:**	Not determined.
· **Danger of explosion:**	Product does not present an explosion hazard.
· **Explosion limits:**	
Lower:	Not determined.
Upper:	Not determined.
· **Vapour pressure:**	Not determined.
· **Density:**	Not determined.
· **Relative density**	Not determined.
· **Vapour density**	Not determined.
· **Evaporation rate**	Not determined.
· **Solubility in / Miscibility with water:**	Not determined.
· **Segregation coefficient (n-octanol/water):**	Not determined.
· **Viscosity:**	
Dynamic:	Not determined.
Kinematic:	Not determined.
· **Other information**	No further relevant information available.

10 Stability and reactivity

· **Reactivity**
· **Chemical stability:** Stable. Avoid strong oxidizing agents.
· **Thermal decomposition / conditions to be avoided:**
No decomposition if used according to specifications.
· **Possibility of hazardous reactions:** No dangerous reactions known.

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according to 1907/2006/EC, Article 31

Printing date 03.02.2011 Revision: 03.02.2011

Trade name: Bafilomycin A1

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· **Contact:** tel. +1 610-941-0430
· **Abbreviations and acronyms:**
IMDG: International Maritime Code for Dangerous Goods
GHS: Globally Harmonized System of Classification and Labelling of Chemicals
CAS: Chemical Abstracts Service (division of the American Chemical Society)

Conforms to 93/112/EC and ISO 11014-1

SAFETY DATA SHEET

CALBIOCHEM®

Order Number 3973698-0

Customer Number 6110504

1. Identification of the substance/preparation and of the company/undertaking

Product name : Phorbol-12-myristate-13-acetate

Chemical formula : $C_{36}H_{56}O_8$

Synonym : PMA; TPA
13-O-Acetylphorbol 12-myristate
Factor A1
Phorbol Acetate, myristate
Phorbol monoacetate monomyristate
Phorbol myristate acetate

Catalog # : 524400

Supplier : EMD Biosciences, Inc.
10394 Pacific Center Court
San Diego, CA 92121
PHONE: (858)450-5558/(800)854-3417
FAX: (858)453-3552

Emergency telephone number : Call Chemtrec®
(800)424-9300 (within U.S.A.)
(703)527-3887 (outside U.S.A.)

2. Composition / information on ingredients

Substance/Preparation : Substance

Chemical name*	CAS No.	EC Number	Symbol	R-Phrases
12-tetradecanoylphorbol-13-acetate	16561-29-8	Not available.	Xn	R21, R38

3. Hazards identification

Physical/chemical hazards : Not applicable.

Human health hazards : CAUTION!
MAY BE HARMFUL IF ABSORBED THROUGH SKIN.
MAY CAUSE SKIN IRRITATION.

4. First-aid measures

First-Aid measures

Inhalation : If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion : Do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a physician immediately. Loosen tight clothing such as a collar, tie, belt or waistband.

Skin Contact : In case of contact, immediately flush skin with plenty of water. Cover the irritated skin with an emollient. Remove contaminated clothing and shoes. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention.

Eye Contact : Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Effects and symptoms

Skin Contact : Hazardous in case of skin contact (irritant, permeator). Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering.

Aggravating conditions : Repeated or prolonged exposure is not known to aggravate medical condition.

5. Fire-fighting measures

Flammability of the Product : May be combustible at high temperature.

Extinguishing Media

Suitable : SMALL FIRE: Use DRY chemical powder.
LARGE FIRE: Use water spray, fog or foam. Do not use water jet.

Hazardous thermal (de)composition products : These products are carbon oxides (CO, CO2).

Special fire-fighting procedures : Fire fighters should wear positive pressure self-contained breathing apparatus (SCBA) and full turnout gear.

Protection of fire-fighters : Be sure to use an approved/certified respirator or equivalent.

6. Accidental release measures

Personal precautions : Splash goggles. Full suit. Dust respirator. Boots. Gloves. A self-contained breathing apparatus should be used to avoid inhalation of the product. Suggested protective clothing might not be sufficient; consult a specialist BEFORE handling this product.

Small Spill and Leak : Use appropriate tools to put the spilled solid in a convenient waste disposal container.

Large Spill and Leak : Use a shovel to put the material into a convenient waste disposal container.

7. Handling and storage

Handling : Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not ingest. Do not breathe dust. Avoid contact with skin. Wear suitable protective clothing. If ingested, seek medical advice immediately and show the container or the label.

Storage : Keep container tightly closed. Keep container in a cool, well-ventilated area. Do not store above -20°C (-4°F).

Packaging materials

Recommended use : Use original container.

8. Exposure controls/personal protection

Engineering measures : Use process enclosures, local exhaust ventilation, or other engineering controls to keep airborne levels below recommended exposure limits. If user operations generate dust, fume or mist, use ventilation to keep exposure to airborne contaminants below the exposure limit.

Hygiene measures : Wash hands, forearms, and face thoroughly after handling compounds and before eating, smoking, using lavatory, and at the end of day.

Ingredient Name	Occupational Exposure Limits
Phorbol-12-myristate-13-acetate	Not available.

Personal protective equipment

Respiratory system : Dust respirator. Be sure to use an approved/certified respirator or equivalent.

Skin and body : Lab coat.

Hands : Gloves.

Eyes : Safety glasses.

Protective Clothing (Pictograms) :

9. Physical and chemical properties

Physical state : Solid.

Color : White.

Molecular Weight : 616.8 g/mole

Melting Point : 50 to 70°C (122 to 158°F)

Solubility : Not available.

Flash point : Not available.

Explosive properties : Risks of explosion of the product in presence of mechanical impact: Not available.
Risks of explosion of the product in presence of static discharge: Not available.

10. Stability and reactivity

Stability : The product is stable.

Conditions to avoid : Not available.

Hazardous Decomposition Products : These products are carbon oxides (CO, CO2).

11. Toxicological information

RTECS # : QH4377000

Local effects

Skin irritation : Hazardous in case of skin contact (irritant).

Acute toxicity : LD50: Not available.
LC50: Not available.

Chronic toxicity : Repeated or prolonged exposure is not known to aggravate medical condition.

Other Toxic Effects on Humans : Not available.
Hazardous in case of skin contact (irritant, permeator).

Not available.

Not available.

Carcinogenic effects : Not available.

Mutagenic effects : Not available.

Reproduction toxicity : Not available.

Teratogenic effects : Not available.

12. Ecological information

Ecotoxicity : Not available.

Toxicity of the Products of Biodegradation : The products of degradation are less toxic than the product itself.

13. Disposal considerations

Methods of disposal: Waste of residues; Contaminated packaging : Waste must be disposed of in accordance with federal, state and local environmental control regulations

14. Transport information

International transport regulations

Land - Road/Railway

ADR/RID Class : Not controlled under ADR (Europe).

Sea

IMDG Class : Not controlled under IMDG.

Air

IATA-DGR Class : Not controlled under IATA.

Special Provisions for Transport : Not applicable.

15. Regulatory information

EU Regulations

Hazard symbol(s) :

Classification : Harmful

Risk Phrases : R21- Harmful in contact with skin.
R38- Irritating to skin.

Safety Phrases : S26- In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S27- Take off immediately all contaminated clothing.
S36- Wear suitable protective clothing.
S45- In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible)

Contains : - 12-tetradecanoylphorbol-13-acetate

U.S. Federal Regulations : TSCA: No products were found.

SARA 302/304/311/312 extremely hazardous substances: No products were found.
SARA 302/304 emergency planning and notification: No products were found.
SARA 302/304/311/312 hazardous chemicals: No products were found.
SARA 311/312 MSDS distribution - chemical inventory - hazard identification: No products were found.
SARA 313 toxic chemical notification and release reporting: No products were found.

Clean Water Act (CWA) 307: No products were found.

Clean Water Act (CWA) 311: No products were found.

Clean air act (CAA) 112 accidental release prevention: No products were found.
Clean air act (CAA) 112 regulated flammable substances: No products were found.
Clean air act (CAA) 112 regulated toxic substances: No products were found.

HCS Classification	:	CLASS: Irritating substance. CLASS: Target organ effects.
State Regulations	:	
WHMIS (Canada)	:	Not controlled under WHMIS (Canada). No products were found.

16. Other information

Hazardous Material Information System (U.S.A.)

	2
	3
Reactivity	0
Personal Protection	E

National Fire Protection Association (U.S.A.)

Notice to Reader

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Final determination of suitability of any material is the sole responsibility of the user. All materials may present unknown hazards and should be used with caution. Although certain hazards are described herein, we cannot guarantee that these are the only hazards that exist. **This product is intended for research use only.**

Conforms to 93/112/EC and ISO 11014-1

SAFETY DATA SHEET

CALBIOCHEM®

Order Number

Customer Number

1. Identification of the substance/preparation and of the company/undertaking

Product name : TAPI-0

Chemical formula : $C_{24}H_{32}N_4O_5$

Synonym : N-(R)-[2-Hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-naphthyla Amide;
TNF-alpha Protease Inhibitor-0

Catalog # : 579050

Supplier : Manufactured by EMD Biosciences, Inc.
10394 Pacific Center Court
San Diego, CA 92121
(858)450-5558/(800)854-3417
FAX: (858)453-3552

Emergency telephone number : Call Chemtrec
(800)424-9300 (within U.S.A.)
(703)527-3887 (outside U.S.A.)

2. Composition / information on ingredients

Substance/Preparation : Substance

Chemical name*	CAS No.	EC Number	Symbol	R-Phrases
N-(R)-[2-Hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-naı Amide; TNF-alpha Protease Inhibitor-0		Not available.	-	-

3. Hazards identification

Physical/chemical hazards : Not applicable.

Human health hazards : No specific hazard.

4. First-aid measures

First-Aid measures

Inhalation : If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion : Do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a physician immediately. Loosen tight clothing such as a collar, tie, belt or waistband.

Skin Contact : In case of contact, immediately flush skin with plenty of water. Remove contaminated clothing and shoes. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention.

Eye Contact : Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Aggravating conditions : Repeated or prolonged exposure is not known to aggravate medical condition.

5. Fire-fighting measures

Flammability of the Product : May be combustible at high temperature.

Extinguishing Media

Suitable : SMALL FIRE: Use DRY chemical powder.
LARGE FIRE: Use water spray, fog or foam. Do not use water jet.

Hazardous thermal (de)composition products : These products are carbon oxides (CO, CO2), nitrogen oxides (NO, NO2...).

Special fire-fighting procedures : Fire fighters should wear positive pressure self-contained breathing apparatus (SCBA) and full turnout gear.

Protection of fire-fighters : Be sure to use an approved/certified respirator or equivalent.

6. Accidental release measures

Personal precautions : Splash goggles. Full suit. Boots. Gloves. Suggested protective clothing might not be sufficient; consult a specialist BEFORE handling this product.

Small Spill and Leak : Use appropriate tools to put the spilled solid in a convenient waste disposal container.

Large Spill and Leak : Use a shovel to put the material into a convenient waste disposal container.

7. Handling and storage

Handling : Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk. evaporate the residue under a fume hood. Ground all equipment containing material. Do not breathe dust.

Storage : Keep container tightly closed. Keep container in a cool, well-ventilated area. Do not store above -20°C (-4°F).

Packaging materials

Recommended use : Use original container.

8. Exposure controls/personal protection

Engineering measures : Use process enclosures, local exhaust ventilation, or other engineering controls to keep airborne levels below recommended exposure limits. If user operations generate dust, fume or mist, use ventilation to keep exposure to airborne contaminants below the exposure limit.

Hygiene measures : Wash hands after handling compounds and before eating, smoking, using lavatory, and at the end of day.

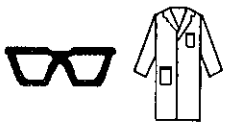
Ingredient Name	Occupational Exposure Limits
TAPI-0	Not available.

Personal protective equipment

Skin and body : Lab coat.

Eyes : Safety glasses.

Protective Clothing (Pictograms) :



9. Physical and chemical properties

Physical state : Solid. (Solid powder.)

Color : White.

Molecular Weight : 456.5 g/mole

Solubility : Not available.

Flash point : Not available.

Explosive properties : Risks of explosion of the product in presence of mechanical impact: Not available.
Risks of explosion of the product in presence of static discharge: Not available.

10. Stability and reactivity

Stability : The product is stable.

Conditions to avoid : Hygroscopic; keep container tightly closed. Sensitive to light.

Hazardous Decomposition Products : These products are carbon oxides (CO, CO2), nitrogen oxides (NO, NO2...).

11. Toxicological information

RTECS # : **Not available.**

Local effects

Skin irritation : Not available.

Acute toxicity : LD50: Not available.
LC50: Not available.

Chronic toxicity : Repeated or prolonged exposure is not known to aggravate medical condition.

Other Toxic Effects on Humans : Not available.
No specific information is available in our database regarding the other toxic effects of this material for humans

Not available.

To the best of our knowledge, the toxicological properties of this product have not been thoroughly investigated.

Carcinogenic effects	:	Not available.
Mutagenic effects	:	Not available.
Reproduction toxicity	:	Not available.
Teratogenic effects	:	Not available.

12. Ecological information

Ecotoxicity	:	Not available.
Toxicity of the Products of Biodegradation	:	The product itself and its products of degradation are not toxic.

13. Disposal considerations

Methods of disposal: Waste of residues; Contaminated packaging	:	Waste must be disposed of in accordance with federal, state and local environmental control regulations.

14. Transport information

International transport regulations

Land - Road/Railway

ADR/RID Class	:	Not controlled under ADR (Europe).

Sea

IMDG Class	:	Not controlled under IMDG.

Air

IATA-DGR Class	:	Not controlled under IATA.
Special Provisions for Transport		Not applicable.

15. Regulatory information

EU Regulations

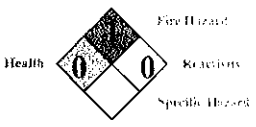
Risk Phrases	:	This product is not classified according to the EU regulations.
U.S. Federal Regulations	:	TSCA: No products were found. SARA 302/304/311/312 extremely hazardous substances: No products were found. SARA 302/304 emergency planning and notification: No products were found. SARA 302/304/311/312 hazardous chemicals: No products were found. SARA 311/312 MSDS distribution - chemical inventory - hazard identification: No products were found. SARA 313 toxic chemical notification and release reporting: No products were found. Clean Water Act (CWA) 307: No products were found. Clean Water Act (CWA) 311: No products were found. Clean air act (CAA) 112 accidental release prevention: No products were found. Clean air act (CAA) 112 regulated flammable substances: No products were found. Clean air act (CAA) 112 regulated toxic substances: No products were found.
HCS Classification	:	Not controlled under the HCS (United States).
State Regulations	:	
WHMIS (Canada)	:	Not controlled under WHMIS (Canada). No products were found.

16. Other information

Hazardous Material Information System (U.S.A.)

	0
Reactivity	0
Personal Protection	A

National Fire Protection Association (U.S.A.)



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Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Gemcitabine Hydrochloride
Proposed Use Dose:	125 µg
Proposed Storage Dose:	50000 µg
LD_{50} (species):	7333000 µg

Calculation:

7333000 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 366650000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = 36665000

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Tunicamycin
Proposed Use Dose:	5 µg
Proposed Storage Dose:	**5000** µg
LD $_{50}$ (species):	400 µg

Calculation:

400 µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg = 20000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = ***2000***

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Cycloheximide
Proposed Use Dose:	100 µg
Proposed Storage Dose:	1000000 µg
LD $_{50}$ (species):	2000 µg

Calculation:

2000 µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg = 100000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = ***10000***

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	MG132
Proposed Use Dose:	1 µg
Proposed Storage Dose:	1000 µg
LD $_{50}$ (species):	Unknown µg

Calculation:

Unknown µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg =

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = *0*

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	ALLN
Proposed Use Dose:	5 µg
Proposed Storage Dose:	5000 µg
LD $_{50}$ (species):	Unknown µg

Calculation:

Unknown µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg =

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = *0*

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Y27632
Proposed Use Dose:	1 µg
Proposed Storage Dose:	1000 µg
LD $_{50}$ (species):	Unknown µg

Calculation:

Unknown µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg =

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = *0*

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Dithiotheitol (DTT)
Proposed Use Dose:	1 µg
Proposed Storage Dose:	**5000000** µg
LD_{50} (species):	400000 µg

Calculation:

400000 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 20000000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = ***2000000***

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Bafilomycin A1
Proposed Use Dose:	1 µg
Proposed Storage Dose:	100 µg
LD_{50} (species):	Unknown µg

Calculation:

Unknown µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg =

LD_{50} per person with safety factor of 10 based on LD_{50} in µg =

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Phorbol-12-myristate-13-acetate (PMA)
Proposed Use Dose:	1 µg
Proposed Storage Dose:	1000 µg
LD $_{50}$ (species):	Unknown µg

Calculation:

Unknown µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg =

LD_{50} per person with safety factor of 10 based on LD_{50} in µg =

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	TAPI-O
Proposed Use Dose:	1 µg
Proposed Storage Dose:	1000 µg
LD_{50} (species):	Unknown µg

Calculation:

Unknown µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg =

LD_{50} per person with safety factor of 10 based on LD_{50} in µg =

Comments/Recommendations:

Modification Form for Permit BIO-UWO-0256

Permit Holder: Lakshman Gunaratnam

Approved Personnel
(Please stroke out any personnel to be removed)

Ola Ismail

Xinghong Xhang

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. Give the full name - do not abbreviate.
Approved Microorganisms	E.coli, Lentivirus	
Approved Primary and Established Cells	Mouse[primary] kidney, spleen, blood, bone marrow. Human[established]786-0, 769-P, HEK293, HEK293T/17, Jurkat, HK-2 Rodent[established] JAWSII, mIMCD-3, CMT-93 Porcine [established]LLC-PK1, canine	DC, 2, 4 (mouse)
Approved Use of Human Source Material		
Approved Genetic Modifications (Plasmids/Vectors)	[plasmids]: pcDNA3. [vectors]: pLUX-puro proprietory	
Approved Use of Animals	Musculus	
Approved Biological Toxin(s)	C3 Exotoxin, Phalloidin	SB202190 (S)-(+)-Camptothecin GM6001 ~~DMSO~~

DMSO does not need to be listed (per march meeting)

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF..*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: _____

Current Classification: 2+ **Containment Level for Added Biohazards:** _____

Date of Last Biohazardous Agents Registry Form: Jun 29, 2010

Date of Last Modification (if applicable): Feb 08, 2011

BioSafety Officer(s): J Stanley April 12, 2011

Chair, Biohazards Subcommittee: _____ **Date:** 14 April 2011

1) Captothecin: Used to induce apoptosis in cells. Apoptotic cells are used as targets for phagocytosis in our experiments.[1]
2) SB 202190: Used to block MEK kinase in our cells. MEK kinase is believed to involved in metalloproease-mediated cleavage of KIM-1.[2]
3) GM6001: Used to block metalloproteases involved in KIM-1 shedding.[2] GM6001 will be injected into mice after ischemia-reperfusion injury surgery. This protocol will be submitted as a modification to our animal protocol.
4) DC 2.4 mouse dendritic cells were acquired from Dr. Kenneth Rock at Dana Farber Cancer Institute (via MTA). They will be used to study the interrelationship of antigen presenting cells with our kidney epithelial cells.

1. Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV. Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. *J Clin Invest.* May 2008;118(5):1657-1668.
2. Zhang Z, Humphreys BD, Bonventre JV. Shedding of the urinary biomarker kidney injury molecule-1 (KIM-1) is regulated by MAP kinases and juxtamembrane region. *J Am Soc Nephrol.* Oct 2007;18(10):2704-2714.

For the Captothecin:
How much (in ug) do you handle at once? 5mM ~~x~~ in 15 uL
How much (in ug) do you store? 100mg
Please provide an LD50. Acute oral toxicity (LD50): 50.1 mg/kg [Mouse]

per email March 23, 2011

For the SB202190:
How much (in ug) do you handle at once? 0.05mg
How much (in ug) do you store? 5mg
Please provide an LD50. Acute dermal toxicity (LD50): 40000 mg/kg [Rat].

For the GM6001:
How much (in ug) do you handle at once? 0.010.05 mg
How much (in ug) do you store? 1mg-1g
Please provide an LD50. LD50: Not available.

0.01-.05 mg per e-mail March 23, 2011

TOXIN USE RISK ASSESSMENT

Name of Toxin:	SB202190
Proposed Use Dose:	50 µg
Proposed Storage Dose:	500 µg
LD $_{50}$ (species):	40000 µg

Calculation:

40000 µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg = 2000000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = 200000

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	Captothecin
Proposed Use Dose:	1.7 µg
Proposed Storage Dose:	10000 µg
LD_{50} (species):	50100 µg

Calculation:

50100 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 2505000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = 250500

Comments/Recommendations:

Material Safety Data Sheet

Version 4.1
Revision Date 10/22/2010
Print Date 02/07/2011

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : (*S*)-(+)-Camptothecin

Product Number : C9911
Brand : Sigma
Product Use : For laboratory research purposes.

Supplier :	Sigma-Aldrich Canada, Ltd 2149 Winston Park Drive OAKVILLE ON L6H 6J8 CANADA	Manufacturer :	Sigma-Aldrich Corporation 3050 Spruce St. St. Louis, Missouri 63103 USA
Telephone :	+19058299500		
Fax :	+19058299292		
Emergency Phone # (For both supplier and manufacturer) :	1-800-424-9300		
Preparation Information :	Sigma-Aldrich Corporation Product Safety - Americas Region 1-800-521-8956		

2. HAZARDS IDENTIFICATION

Emergency Overview

WHMIS Classification

D1B Toxic Material Causing Immediate and Serious Toxic Effects — Toxic by ingestion

GHS Classification

Acute toxicity, Oral (Category 3)

GHS Label elements, including precautionary statements

Pictogram

Signal word Danger

Hazard statement(s)

H301	Toxic if swallowed.

Precautionary statement(s)

P264	Wash skin thoroughly after handling.
P270	Do not eat, drink or smoke when using this product.
P301 + P310	IF SWALLOWED: Immediately call a POISON CENTER or doctor/ physician.
P321	Specific treatment (see supplemental first aid instructions on this label).
P330	Rinse mouth.
P405	Store locked up.
P501	Dispose of contents/ container to an approved waste disposal plant.

HMIS Classification

Health hazard:	2
Flammability:	0
Physical hazards:	0

Potential Health Effects

Inhalation May be harmful if inhaled. May cause respiratory tract irritation.

Skin	May be harmful if absorbed through skin. May cause skin irritation.
Eyes	May cause eye irritation.
Ingestion	Toxic if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Formula : $C_{20}H_{16}N_2O_4$
Molecular Weight : 348.35 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
(S)-(+)-Camptothecin			
7689-03-4	-	-	-

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.

In case of eye contact
Flush eyes with water as a precaution.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIRE-FIGHTING MEASURES

Conditions of flammability
Not flammable or combustible.

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)

Explosion data - sensitivity to mechanical impact
no data available

Explosion data - sensitivity to static discharge
no data available

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Wear respiratory protection. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Avoid breathing dust.

Environmental precautions
Prevent further leakage or spillage if safe to do so. Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: 2 - 8 °C

Keep in a dry place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
Where risk assessment shows air-purifying respirators are appropriate use a full-face particle respirator type N99 (US) or type P2 (EN 143) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Face shield and safety glasses Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
Complete suit protecting against chemicals, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Avoid contact with skin, eyes and clothing. Wash hands before breaks and immediately after handling the product.

Specific engineering controls
Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	powder
Colour	no data available

Safety data

pH	no data available
Melting/freezing point	Melting point/range: 260 °C (500 °F) - dec.
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available

Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
LD50 Oral - rat - 153 mg/kg

Inhalation LC50
no data available

Dermal LD50
no data available

Other information on acute toxicity
no data available

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization
no data available

Germ cell mutagenicity

Genotoxicity in vitro - mouse - lymphocyte
Other mutation test systems

Genotoxicity in vitro - Hamster - ovary
Sister chromatid exchange

Genotoxicity in vitro - Human - leukocyte
DNA inhibition

Genotoxicity in vitro - Hamster - Lungs
Mutation in mammalian somatic cells.

Genotoxicity in vitro - Hamster - Lungs

Sister chromatid exchange

Genotoxicity in vitro - mouse - leukocyte
DNA inhibition

Genotoxicity in vitro - Hamster - ovary
DNA damage

Genotoxicity in vitro - Human - HeLa cell
DNA inhibition

Genotoxicity in vitro - Chicken - Embryo
DNA inhibition

Genotoxicity in vitro - Human - HeLa cell
Other mutation test systems

Genotoxicity in vitro - Human - leukocyte
Other mutation test systems

Genotoxicity in vitro - Human - lymphocyte
Sister chromatid exchange

Genotoxicity in vitro - Hamster - Lungs
Cytogenetic analysis

Genotoxicity in vitro - Hamster - ovary
Cytogenetic analysis

Genotoxicity in vitro - Hamster - Lungs
DNA damage

Genotoxicity in vitro - mouse - lymphocyte
DNA inhibition

Genotoxicity in vitro - mouse - leukocyte
DNA damage

Genotoxicity in vitro - Monkey - Kidney
DNA damage

Genotoxicity in vitro - Human - lymphocyte
Cytogenetic analysis

Genotoxicity in vitro - Human - Other cell types
Cytogenetic analysis

Genotoxicity in vitro - Human - fibroblast
DNA damage

Genotoxicity in vitro - Human - HeLa cell
DNA damage

Genotoxicity in vitro - Human - lymphocyte
DNA damage

Genotoxicity in vitro - Human - Other cell types
DNA damage

Genotoxicity in vivo - mouse - Intraperitoneal
Cytogenetic analysis

Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
no data available

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. May cause respiratory tract irritation.
Ingestion	Toxic if swallowed.
Skin	May be harmful if absorbed through skin. May cause skin irritation.
Eyes	May cause eye irritation.

Signs and Symptoms of Exposure
To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Synergistic effects
no data available

Additional Information
RTECS: UQ0492000

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
UN-Number: 1544 Class: 6.1 Packing group: III
Proper shipping name: Alkaloids, solid, n.o.s. ((S)-(+)-Camptothecin)
Marine pollutant: No

Poison Inhalation Hazard: No

IMDG
UN-Number: 1544 Class: 6.1 Packing group: III EMS-No: F-A, S-A
Proper shipping name: ALKALOIDS, SOLID, N.O.S. ((S)-(+)-Camptothecin)
Marine pollutant: No

IATA
UN-Number: 1544 Class: 6.1 Packing group: III
Proper shipping name: Alkaloids, solid, n.o.s. ((S)-(+)-Camptothecin)

15. REGULATORY INFORMATION

DSL Status
This product contains the following components that are not on the Canadian DSL nor NDSL lists.

	CAS-No.
(S)-(+)-Camptothecin	7689-03-4

WHMIS Classification

D1B	Toxic Material Causing Immediate and Serious Toxic Effects	Toxic by ingestion

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Conforms to 93/112/EC and ISO 11014-1

SAFETY DATA SHEET

CALBIOCHEM®

Order Number **Customer Number**

1. Identification of the substance/preparation and of the company/undertaking

Product name : **GM6001 in Solution**

Chemical formula : $C_{20}H_{28}N_4O_4$

Synonym : Not available.

Catalog # : 364206

Supplier : EMD Biosciences, Inc.
10394 Pacific Center Court
San Diego, CA 92121
(858)450-5558/(800)854-3417
FAX: (858)453-3552

Emergency telephone number : Call Chemtrec®
(800)424-9300 (within U.S.A.)
(703)527-3887 (outside U.S.A.)

2. Composition / information on ingredients

Substance/Preparation : Substance

Chemical name*	CAS No.	EC Number	Symbol	R-Phrases
GM6001 in Solution		Not available.	Xi	R36/38

3. Hazards identification

Physical/chemical hazards : Not applicable.

Human health hazards : CAUTION!
MAY CAUSE EYE AND SKIN IRRITATION.

4. First-aid measures

First-Aid measures

Inhalation : If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion : Do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a physician immediately. Loosen tight clothing such as a collar, tie, belt or waistband.

Skin Contact : In case of contact, immediately flush skin with plenty of water. Cover the irritated skin with an emollient. Remove contaminated clothing and shoes. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention.

Eye Contact : Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Effects and symptoms

Skin Contact : Hazardous in case of skin contact (irritant). Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering.

Eye Contact : Hazardous in case of eye contact (irritant).

Aggravating conditions : Repeated or prolonged exposure is not known to aggravate medical condition.

5. Fire-fighting measures

Flammability of the Product : May be combustible at high temperature.

Extinguishing Media

Suitable : SMALL FIRE: Use DRY chemical powder.
LARGE FIRE: Use water spray, fog or foam. Do not use water jet.

Hazardous thermal (de)composition products : These products are carbon oxides (CO, CO2), nitrogen oxides (NO, NO2...).

Special fire-fighting procedures : Fire fighters should wear positive pressure self-contained breathing apparatus (SCBA) and full turnout gear.

Protection of fire-fighters : Be sure to use an approved/certified respirator or equivalent.

6. Accidental release measures

Personal precautions : Splash goggles. Full suit. Boots. Gloves. Suggested protective clothing might not be sufficient; consult a specialist BEFORE handling this product.

Small Spill and Leak : Absorb with an inert material and put the spilled material in an appropriate waste disposal.

Large Spill and Leak : Absorb with an inert material and put the spilled material in an appropriate waste disposal.

7. Handling and storage

Handling : Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not breathe gas/fumes/ vapor/spray. Wear suitable protective clothing. If you feel unwell, seek medical attention and show the label when possible. Avoid contact with skin and eyes.

Storage : Keep container tightly closed. Keep container in a cool, well-ventilated area. Do not store above 4°C (39.2°F).

Packaging materials

Recommended use : Use original container.

8. Exposure controls/personal protection

Engineering measures : Provide exhaust ventilation or other engineering controls to keep the airborne concentrations of vapors below their respective threshold limit value. Ensure that eyewash stations and safety showers are proximal to the work-station location.

Hygiene measures : Wash hands after handling compounds and before eating, smoking, using lavatory, and at the end of day.

Ingredient Name	Occupational Exposure Limits
GM6001 in Solution	Not available.

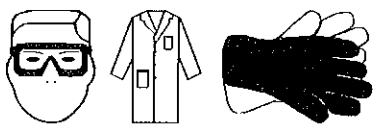
Personal protective equipment

Skin and body : Lab coat.

Hands : Gloves.

Eyes : Splash goggles.

Protective Clothing (Pictograms) :



9. Physical and chemical properties

Physical state : Liquid. (Supplied in DMSO)

Color : Not available.

Molecular Weight : 388.5 g/mole

Solubility : Not available.

Flash point : Not available.

Explosive properties : Risks of explosion of the product in presence of mechanical impact: Not available.
Risks of explosion of the product in presence of static discharge: Not available.

10. Stability and reactivity

Stability : The product is stable.

Conditions to avoid : Hygroscopic; keep container tightly closed.

Hazardous Decomposition Products : These products are carbon oxides (CO, CO2), nitrogen oxides (NO, NO2...).

11. Toxicological information

RTECS # : **Not available.**

Local effects

Skin irritation : Hazardous in case of skin contact (irritant).

Acute toxicity : LD50: Not available.
LC50: Not available.

Chronic toxicity : Repeated or prolonged exposure is not known to aggravate medical condition.

Other Toxic Effects on Humans : Not available.
Hazardous in case of skin contact (irritant), of eye contact (irritant).

Not available.

To the best of our knowledge, the toxicological properties of this product have not been thoroughly investigated

Carcinogenic effects	:	Not available
Mutagenic effects	:	Not available.
Reproduction toxicity	:	Not available
Teratogenic effects	:	Not available.

12. Ecological information

Ecotoxicity	:	Not available.
Toxicity of the Products of Biodegradation	:	The product itself and its products of degradation are not toxic.

13. Disposal considerations

Methods of disposal: Waste of residues; Contaminated packaging	:	Waste must be disposed of in accordance with federal, state and local environmental control regulations.

14. Transport information

International transport regulations

Land - Road/Railway

ADR/RID Class	:	Not controlled under ADR (Europe).

Sea

IMDG Class	:	Not controlled under IMDG.

Air

IATA-DGR Class	:	Not controlled under IATA.
Special Provisions for Transport		Not applicable.

15. Regulatory information

EU Regulations

Hazard symbol(s) :

Classification	:	Irritant
Risk Phrases	:	R36/38- Irritating to eyes and skin.
Safety Phrases	:	S26- In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

U.S. Federal Regulations :

TSCA: No products were found.
SARA 302/304/311/312 extremely hazardous substances: No products were found.
SARA 302/304 emergency planning and notification: No products were found.
SARA 302/304/311/312 hazardous chemicals: No products were found.
SARA 311/312 MSDS distribution - chemical inventory - hazard identification: No products were found.
SARA 313 toxic chemical notification and release reporting: No products were found.
Clean Water Act (CWA) 307: No products were found.
Clean Water Act (CWA) 311: No products were found.
Clean air act (CAA) 112 accidental release prevention: No products were found.
Clean air act (CAA) 112 regulated flammable substances: No products were found.
Clean air act (CAA) 112 regulated toxic substances: No products were found.

HCS Classification	:	CLASS: Irritating substance.
State Regulations	:	

WHMIS (Canada) : Not controlled under WHMIS (Canada).

No products were found.

16. Other information

Hazardous Material Information System (U.S.A.)

	2
	1
Reactivity	0
Personal Protection	J

National Fire Protection Association (U.S.A.)

Notice to Reader

Material Safety Data Sheet

Version 4.1
Revision Date 10/21/2010
Print Date 02/07/2011

1. PRODUCT AND COMPANY IDENTIFICATION

Product name	: SB 202190			
Product Number	: S7067			
Brand	: Sigma			
Product Use	: For laboratory research purposes.			
Supplier	: Sigma-Aldrich Canada, Ltd 2149 Winston Park Drive OAKVILLE ON L6H 6J8 CANADA	Manufacturer	:	Sigma-Aldrich Corporation 3050 Spruce St. St. Louis, Missouri 63103 USA
Telephone	: +19058299500			
Fax	: +19058299292			
Emergency Phone # (For both supplier and manufacturer)	: 1-800-424-9300			
Preparation Information	: Sigma-Aldrich Corporation Product Safety - Americas Region 1-800-521-8956			

2. HAZARDS IDENTIFICATION

Emergency Overview

WHMIS Classification

D2B	Toxic Material Causing Other Toxic Effects	Moderate skin irritant Moderate respiratory irritant Moderate eye irritant

GHS Classification

Skin irritation (Category 2)
Eye irritation (Category 2A)
Specific target organ toxicity - single exposure (Category 3)

GHS Label elements, including precautionary statements

Pictogram

Signal word: Warning

Hazard statement(s)

H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.

Precautionary statement(s)

P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P264	Wash skin thoroughly after handling.
P271	Use only outdoors or in a well-ventilated area.
P280	Wear protective gloves/ eye protection/ face protection.
P302 + P352	IF ON SKIN: Wash with plenty of soap and water.
P304 + P340	IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.
P305 + P351 + P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

P312	Call a POISON CENTER or doctor/ physician if you feel unwell.
P321	Specific treatment (see supplemental first aid instructions on this label).
P332 + P313	If skin irritation occurs: Get medical advice/ attention.
P337 + P313	If eye irritation persists: Get medical advice/ attention.
P362	Take off contaminated clothing and wash before reuse.
P403 + P233	Store in a well-ventilated place. Keep container tightly closed.
P405	Store locked up.
P501	Dispose of contents/ container to an approved waste disposal plant.

HMIS Classification

Health hazard:	2
Flammability:	0
Physical hazards:	0

Potential Health Effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.
Ingestion	May be harmful if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole

Formula : $C_{20}H_{14}FN_3O$

Molecular Weight : 331.34 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
SB 202190			
152121-30-7	-	-	-

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Consult a physician.

In case of eye contact
Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIRE-FIGHTING MEASURES

Conditions of flammability
Not flammable or combustible.

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Hydrogen fluoride

Explosion data - sensitivity to mechanical impact
no data available

Explosion data - sensitivity to static discharge
no data available

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use personal protective equipment. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Avoid breathing dust.

Environmental precautions
Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: 2 - 8 °C

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
For nuisance exposures use type P95 (US) or type P1 (EU EN 143) particle respirator.For higher level protection use type OV/AG/P99 (US) or type ABEK-P2 (EU EN 143) respirator cartridges. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Safety glasses with side-shields conforming to EN166 Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
impervious clothing, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

Specific engineering controls
Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid
Colour	no data available

Safety data

pH	no data available

Melting/freezing point	no data available
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available
Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Hydrogen fluoride

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
no data available

Inhalation LC50
Dermal LD50
no data available

Other information on acute toxicity
no data available

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization

no data available

Germ cell mutagenicity
no data available

Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
Inhalation - May cause respiratory irritation.

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Ingestion	May be harmful if swallowed.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.

Signs and Symptoms of Exposure
To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Synergistic effects
no data available

Additional Information
RTECS: Not available

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

DSL Status
This product contains the following components that are not on the Canadian DSL nor NDSL lists.

	CAS-No.
SB 202190	152121-30-7

WHMIS Classification

D2B	Toxic Material Causing Other Toxic Effects	Moderate skin irritant Moderate respiratory irritant Moderate eye irritant

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

DC 2-4

Cloned Dendritic Cells Can Present Exogenous Antigens on Both MHC Class I and Class II Molecules[1]

Zhenhai Shen,*‡ Glen Reznikoff,† Glenn Dranoff,† and Kenneth L. Rock[2]*‡

Pathways for presenting proteins from the extracellular fluids on MHC class I molecules have been described in macrophages. However, it is uncertain whether similar mechanisms exist in dendritic cells, because conventional preparations of these cells can be contaminated with macrophages. We addressed this issue by transducing granulocyte-macrophage CSF into bone marrow cultures followed by supertransfection with *myc* and *raf* oncogenes. These immortalized clones displayed dendritic morphology, and many expressed the dendritic cell-specific markers DEC-205 and 33D1 as well as high levels of MHC molecules and costimulatory molecules. Using these cloned dendritic cells, we found that exogenous OVA could be presented on both their MHC class I and class II molecules. This presentation was markedly enhanced when the Ag was particulate and internalized by phagocytosis. Presentation of particulate OVA on MHC class I molecules was insensitive to the weak base chloroquine, but was blocked by peptide aldehyde inhibitors of the proteasome, indicating that the class I-presented peptides were generated in the cytosol. Brefeldin A, which inhibits the exocytosis of newly synthesized proteins from the endoplasmic reticulum, also inhibited Ag presentation. These results establish that dendritic cells can present exogenous Ags on MHC class I molecules and appear to use a similar phagosome to cytosol pathway as macrophages. Therefore, dendritic cells are likely to play an important role in generating immune responses to tissue transplants and tumors in vivo. Furthermore, these findings provide an approach for targeting vaccine Ags into these cells to prime immune responses in vivo. *The Journal of Immunology,* **1997, 158: 2723–2730.**

MHC class I molecules play an important role in immune surveillance by displaying antigenic peptides on the cell surface (1). The majority of these presented peptides are generated by a multicatalytic proteolytic particle, the proteasome, which is present in the cytosol and nucleus of all eukaryotic cells (2). These antigenic fragments are then translocated into the endoplasmic reticulum (ER)[3] by the transporter associated with Ag presentation (TAP), where they bind to newly assembled MHC class I molecules and then are transported to the plasma membrane (1). As a consequence of these mechanisms, class I-presented peptides are derived in most situations exclusively from cellular and viral proteins synthesized by the APCs. Ags in the extracellular fluids do not gain access to this pathway in most cells and fail to be presented (3).

However, a subset of APCs can present exogenous Ags on class I molecules (4, 5), and this process is markedly enhanced when the Ag is internalized by phagocytosis (6, 7) or macropinocytosis (8). In these cells the exogenous Ag is transferred from the endocytic compartment into the cytosol where it is degraded and presented by the classical MHC class I pathway (8–10). Alternatively, in some cases peptides from the exogenous Ag appear to be generated in the endocytic compartment and to bind to MHC class I molecules on the cell surface (7, 11–13).

The APCs that can present exogenous Ags on MHC class I are quantitatively recovered from lymphoid tissues in a low density fraction that is enriched in macrophages and dendritic cells (DC) (5). That macrophages can mediate this form of Ag presentation has been shown in assays with highly purified macrophages and cloned macrophage cell lines (14). It has been more difficult to address whether DC possess a similar Ag-presenting capability because purified preparations of these cells are often contaminated with macrophages. Therefore, this question can only be resolved with absolutely pure DC. This is an important issue because DC are extremely potent APCs that are believed to play a key role in the initiation of T cell responses (15). This was the rationale in the present study for isolating cloned dendritic cell lines.

The exogenous pathway is active in vivo (6, 16) and probably plays an important role in generating CTL responses in several situations. A major pathway for stimulating CTL to tumors and transplanted tissue involves the representation of tumor or alloantigens on host bone marrow-derived APCs (17–19). Moreover, a similar pathway may be involved in generating anti-viral responses (3). Finally, this pathway can be exploited for stimulating T cell responses to Ags in vaccines (20). Therefore, it is important to elucidate the underlying cellular and molecular mechanisms underlying this pathway of presentation.

Materials and Methods

Cell lines and Abs

NIH J2 Leuk was provided by Dr. U. Rapp (Wurzburg, Germany) (21). FDCP-1 is a GM-CSF-dependent cell line used to measure GM-CSF production (22). The retrovirus-producing cell line Crip MFG-murine GM-CSF (23), the macrophage cell line A3.1A7 (14), and T-T hybridomas RF33.70 (anti-OVA, K^b) (24) and MF2.2D9 (anti-OVA$^+$ IA^b) (6) were

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[2] Address correspondence and reprint requests to Dr. Kenneth L. Rock, Division of Lymphocyte Biology, Dana Farber Cancer Institute, 44 Binney St., Boston, MA 02115.

[3] Abbreviations used in this paper: ER, endoplasmic reticulum; TAP, transporter associated with antigen presentation; DC, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; RT-PCR, reverse transcription-polymerase chain reaction; LLnL, *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal; LLM, *N*-acetyl-L-leucinyl-L-leucinyl-methional.

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previously described. mAbs Y3 (anti-H2-K^b) (25), Y3P (anti-IA) (26), 2.4G2 (anti-FcγRII) (27), 16-10A1 (anti-B7-1) (28), GL-1 (anti-B7-2) (29), YN1/1.7.4 (anti-ICAM-1) (30), D7 (anti-Ly6A/E) (31), M5/49 (anti-Thy-1) (32), M1/70 (anti-Mac-1) (33), NLDC145 (anti-DEC-205) (34), 33D1 (35), and Monia-2 (36) were provided by the laboratories of origin and/or obtained from the American Type Culture Collection (Rockville, MD).

Immortalization of DC

Bone marrow cells flushed from the femurs and tibias of C57BL/6 mice (The Jackson Laboratories, Bar Harbor, ME) were depleted of RBC by ammonium chloride treatment. Nucleated cells (5×10^6) were then infected with an amphotropic, replication defective, retrovirus-expressing murine GM-CSF (CRIP MEG-murine GM-CSF) by cocultivation for 48 h in the presence of polybrene as previously described (37). Nonadherent cells were displaced by gentle pipetting and then placed into culture in 24-well dishes in the presence of RPMI, 10% FCS, and 10 ng/ml murine GM-CSF. Cells were refed every 2 days. Cultures developed an adherent monolayer and clusters of DC colonies. Cultures were dispersed when confluent and placed into medium lacking GM-CSF. After an additional 2 wk in culture in the absence of GM-CSF to expand the cell population, the floating cells were collected and infected with a retrovirus encoding *myc* and *raf* using supernatant (50%) from NIH J2 Leuk cells. After 36 h at 37°C, floating cells were collected, washed, and resuspended in DMEM high glucose supplemented with 10% FCS, L-glutamine, and antibiotics and passaged in tissue culture flasks. From these cell lines DC were subcloned by limiting dilution. For the subcloning and initial passage, conditioned media (50%) from the uncloned DC lines was added to the culture medium.

Immunofluorescence

Immunofluorescence staining was performed as previously described (38). Briefly, dendritic clones were incubated with mAb-containing supernatants for 45 min at 4°C, followed by FITC-conjugated goat anti-rat IgG or FITC-conjugated goat anti-mouse IgG (1/40; Cappel, Organon Teknika Corp., West Chester, PA). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson).

RNA extraction and RT-PCR

Total RNA was extracted from cells and purified using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA), and cDNA was prepared using reverse transcriptase. The oligonucleotide primers CCTTTGTGCCCAGCCT TATA (complementary to the positive strand of DEC-205 sequence, position 4781) and CATTCTTTTCCCAGTTACCT (complementary to the negative strand of DEC-205 sequence, position 685) were synthesized by the Molecular Biology Core Facility of Dana-Farber Cancer Institute. A plasmid containing 5′ 2-kb DEC-205 cDNA (kindly provided by Drs. Wanping Jiang and Michel Nussenzweig, Rockefeller University, New York, NY) and cDNA from various clones were used as templates in PCR reaction. PCR-amplified products were analyzed on a 1.2% agarose gel.

Assays for phagocytosis

For ultrastructural analysis, DC2.4 cells were incubated with latex beads (3 μm in diameter) in 10-cm diameter culture dishes at 37°C for 30 min, and then washed and fixed with 1% paraformaldehyde. Subsequent embedding, ultrathin sectioning, and electron microscopy were performed at the Core Facility of Harvard Medical School (Boston, MA). For immunofluorescence analysis, cells were plated on coverslips, incubated with FITC-conjugated latex beads (3 μm in diameter) at 37°C for 30 min, and washed. Cells were examined on a fluorescence microscope (with the help of Dr. Joel Swanson, Harvard Medical School), and trypan blue (Fisher Scientific, Pittsburgh, PA; 25°C, pH 5.0) was added to quench extracellular FITC-conjugated beads.

Preparation of Ag beads

Iron oxide beads (from PerSeptive Diagnostics, Cambridge, MA) were covalently conjugated to chicken egg OVA according to manufacturer's instruction. FITC (Sigma Chemical Co., St. Louis, MO) was passively absorbed to Latex beads (Polyscience, Inc., Warrington, PA; 1 μm in diameter) according to the manufacturer's instructions.

Ag presentation assays

APC were incubated in serum-free OptiMEM (Life Technologies, Gaithersburg, MD) supplemented with Nutridoma (Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C with or without the inhibitors brefeldin A (5 μg/ml, Sigma Chemical Co.), chloroquine (100 μM; Sigma Chemical Co.), cytochalasin B (5 μg/ml; Sigma Chemical Co.), LLnL (40 μM), LLM (40 μM), and MG132 (10 μM), followed by the addition of Ag. Ag was added in soluble form or bound to iron oxide beads. In some cases, OVA was loaded onto the cytosol by osmotic lysis of pinosomes (39), or SIIN FEKL was expressed in the cytosol using a vaccinia recombinant (a gift from Drs. Jon Yewdell and Jack Bennink) (40). After 5-h incubation at 37°C, the cells were washed, fixed with 1% paraformaldehyde solution, and added to microtiter plates. In some assays, live APCs (10^5/well) were incubated with varying concentrations of Ags in 200 μl of culture medium in flat-bottom microtiter plates. The culture medium was RPMI 1640 prepared as previously described and containing 0.25 μM indomethacin (41). Specific T-T hybrids were added to the microtiter plates and incubated for 20 h at 37°C, after which an aliquot (100 μl) of supernatant was collected and freeze-thawed. The IL-2 content in culture supernatants was assayed using an IL-2-dependent cell line CTLL as previously described (42, 43).

Results

Isolation of cell lines with DC morphology

It is well established that bone marrow-derived DC can be cultured in GM-CSF for short periods (44, 45). We attempted to develop long term cell lines by transducing bone marrow cells with GM-CSF. This resulted in the growth of cell populations that contained a subpopulation of cells with dendritic morphology. However, growth of these DC was not sustained beyond 6 wk, and we were unable to clone them.

We, therefore, modified this approach by superinfecting GM-CSF-transduced bone marrow cells with *myc* and *raf* oncogenes. The resulting cell populations contained cells with dendritic morphology that continued to grow in culture. By limiting dilution, 20 clones of DC were obtained in 4 to 5 wk. These cells had prominent dendritic processes and ruffled edges (Fig. 1*A*) that were not observed on macrophages (Fig. 1*B*); they attached to plastic and then detached over time, so that cultures contained both adherent and floating cells. Where examined, the DC clones do not continue to make measurable GM-CSF using a sensitive bioassay (data not shown). These DC clones have been in culture for >6 mo and can be frozen with cryopreservatives and thawed with good viability.

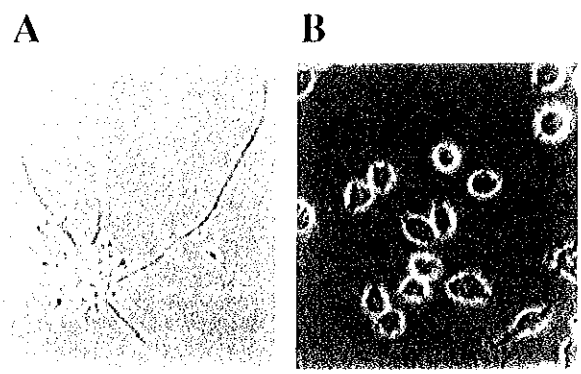

FIGURE 1. Photomicrographs of DC (*A*; DC2.4) and macrophage cells (*B*; A3.1A7). Magnification, ×630.

Characterization of cell surface molecules

We characterized the surface phenotype of these DC clones by immunofluorescence and flow cytometry. Individual fluorescence histograms for one clone are shown in Figure 2 (*A–K*), and the phenotypes of several different clones are summarized in Table I. All clones lacked T cell-specific (e.g., CD3) and B cell-specific (surface Ig) markers (data not shown). Most of these cells expressed high levels of MHC class I and class II molecules, the costimulatory molecules B7-1 and B7-2, as well as CD32 (FcγRII)

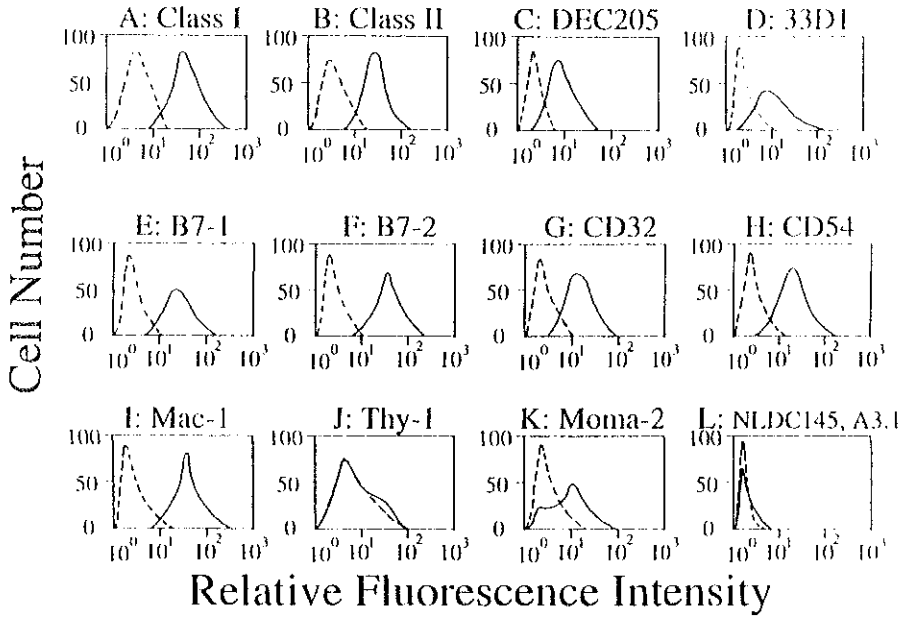

FIGURE 2. Immunofluorescence analysis of immortalized DC 2.4 cell line. DC 2.4 cells (*A–K*) and A3.1A7 macrophages (*L*) were stained by indirect immunofluorescence with mAb supernatants of the indicated specificities followed by appropriate FITC-conjugated secondary Abs as described in *Materials and Methods*. Dotted lines represent cell staining with FITC-conjugated secondary Abs only.

Table I. *Summary of surface phenotype of DC clones and A3.1A7*[a]

Marker	DC				
	DC1.2	DC2.4	DC2.5.1	DC4.1	A3.1A7
DEC205	+	+		+	
33D1	+/-	+		+	
B7-1	+ +	+	+	+	+/-
B7-2	+ +	+ +	+ +	+ +	+ +
Class I	+ +	+ +	+ +	+ +	+ +
Class II	+ +	+	+ +	+	+
CD32	+	+	+	+	+ +
Moma-2	+	+	+	+	+ +
Mac-1	+ +	+	+	+	+ + +
Thy-1					+/-
Ly 6A E		+			+
CD54	+ +	+ +	+ +	+ +	ND

[a] Summary of surface phenotype of representative clones of DC compared with that of macrophage cells, A3.1A7. Four representative DC clones and A3.1A7 were stained by indirect immunofluorescence with mAb supernatants of the indicated specificities followed by appropriate FITC-conjugated secondary Abs as described in *Materials and Methods*. Relative fluorescence intensity is indicated with plus and minus signs; one plus represents approximately one log scale.

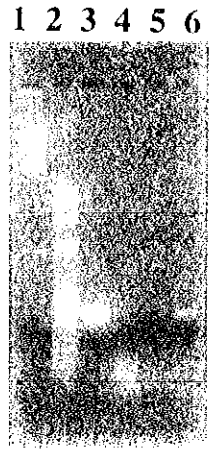

FIGURE 3. Analysis of RT-PCR products on 1.2% agarose gel. *Lane 1*, λ DNA *Hin*dIII digest; *lane 2*, PhiX174 RF DNA *Hae*III digest; *lane 3*, PCR product using plasmid containing DEC-205 cDNA as template; *lane 4*, RT-PCR product of mRNA from A3.1A7 macrophages; *lane 5*, RT-PCR product of mRNA from DC 2.5.1; *lane 6*, RT-PCR product of mRNA from DC 2.4.

and CD54 (intracellular adhesion molecule-1). These markers have all been reported on cultured DC (46–48), although they can also be expressed on other cell types.

The most specific markers for murine DC are the DEC-205 molecule recognized by the mAb NLDC145 (49) and the Ag detected by the mAb 33D1 (35). NLDC145 stained many of the DC clones, e.g., DC1.2, DC2.4, and DC4.1 (Fig. 2). The expression of DEC-205 in these cells was confirmed by RT-PCR (Fig. 3). The other dendritic marker, 33D1, also stained several clones. As expected, the macrophage cells A3.1A7 did not express DEC-205 (Fig. 2*L*) or 33D1 (data not shown). We concluded from these criteria that these cells represented cloned DC.

There were some lines with dendritic morphology that lacked the expression of both dendritic markers (Table I). For 33D1 this might simply reflect the sensitivity of the immunofluorescence assay because even on the DC where we detected expression, the intensity of staining was weak. However, these cells were also negative for the other dendritic marker, DEC-205, by both immunofluorescence and RT-PCR. The DEC-205-positive and -negative cells otherwise appeared to express the same surface molecules and were indistinguishable from one another on morphologic criteria. It is possible that the DEC-205-negative cells are of another lineage of cells (e.g., monocytes) or that there is heterogeneity in the expression of DEC-205 and 33D1 by primary isolated DC.

FIGURE 4. Ultrastructure of DC2.4 incubated with 3 μm-diameter Latex beads for 30 min at 37°C and then washed and handled as described in *Materials and Methods*. Magnification, ×8000.

DC clones are phagocytic

To determine whether the DC clones were phagocytic, they were incubated with latex beads. When viewed by phase microscopy, the DC appeared to rapidly internalize these particles (data not shown). To determine whether the beads were ingested and not simply bound to the cell surface, we initially performed ultrastructural studies. Figure 4 shows an electron micrograph of DC2.4 cells incubated with latex beads at 37°C for 30 min. Numerous beads were visualized in most cells and appeared to be in vesicles. However, it is possible that surface-bound beads could give a similar appearance, depending on the plane of sectioning. Therefore, to further verify that beads were internalized into cells, we performed an immunofluorescence analysis. The DC were incubated with FITC-labeled latex beads and then analyzed by fluorescence microscopy. Trypan blue was added to quench the fluorescence of beads outside the cells. Figure 5 showed both phase (Fig. 5*A*) and fluorescence (Fig. 5*B*) images of representative cells. Most DC contained beads whose fluorescence was not quenched with trypan blue. The fluorescence of surface-bound or free beads was quenched. These analyses indicated that the DC clones are phagocytic.

Presentation of OVA on MHC class I and class II molecules

The availability of cloned DC allowed us to examine their Ag-presenting capabilities and particularly whether they could present exogenous Ags on MHC class I molecules. The presentation of Ag was determined by measuring the production of IL-2 from T-T hybrids specific for OVA peptides bound to MHC class I molecules. DC2.4 cells, a representative DC clone, presented on MHC class I molecules both soluble OVA and particulate OVA added to the culture medium (Fig. 6*A*). However, compared with soluble OVA, the presentation of bead-bound OVA was 100- to 1000-fold more efficient (Fig. 6*A*). Other DC clones were similarly capable of presenting OVA on MHC class I molecules (Fig. 6*C*). This

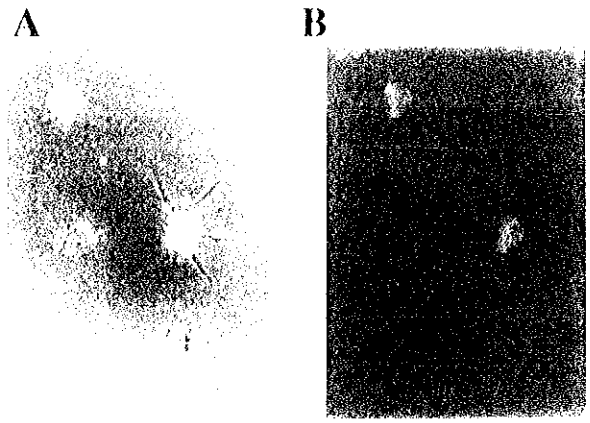

FIGURE 5. DC2.4 are phagocytic. Cells were incubated with 3-μm diameter Latex beads conjugated with FITC for 30 min at 37°C and then quenched with trypan blue (25%, pH 5). Both phase (*A*) and fluorescence (*B*) pictures of the same field were taken. Many intracellular fluorescent beads are of the plane of focus. Extracellular beads were not fluorescent in any plane of focus. Magnification, ×630.

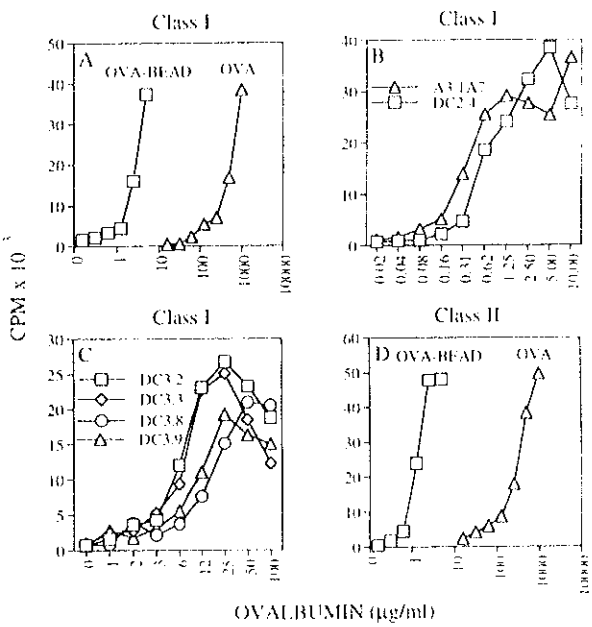

FIGURE 6. Presentation of exogenous OVA and soluble OVA on MHC class I and class II molecules. APCs (5×10^3/well) and RF33.70 (*A*, *B*, and *C*; 5×10^4/well) or ME.2.1D9 (*D*; 5×10^3/well) and the indicated amount of bead-conjugated OVA or soluble OVA were cultured in microliter plates (200 μl). Cultures were then handled as described in *Materials and Methods*. *A*, Presentation of bead-conjugated OVA (open square) and soluble OVA (open triangle) by DC2.4 cells on MHC class I molecules; *B*, presentation of bead-conjugated OVA by DC2.4 (open square) and A3.1A7 (open triangle) on MHC class I molecules. *C*, Presentation of bead-conjugated OVA by various DC clones on MHC class I molecules. *D*, Presentation of bead-conjugated OVA (open square) and soluble OVA (open triangle) by DC2.4 cells on MHC class II molecules.

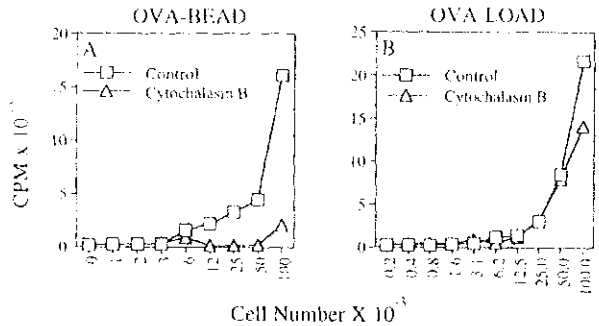

FIGURE 7. Effect of cytochalasin B on exogenous OVA presentation on MHC class I molecules. DC2.4 cells (2×10^6/ml) were pretreated with 5 μg/ml cytochalasin B for 30 min (open triangle) or medium alone (open square) followed by the addition of bead-conjugated OVA (50 μg/ml; *A*) or hypotonic loaded OVA by lysis of pinosome (2 mg/ml; *B*), further incubated for 6 h, and then fixed with 1% paraformaldehyde. Cells were then titrated in microtiter plates (100 μl), and the T-T hybrid cells (5×10^4/well), RF33.70 (*A* and *B*), were added to the cultures (200 μl total) and handled as described in *Materials and Methods*.

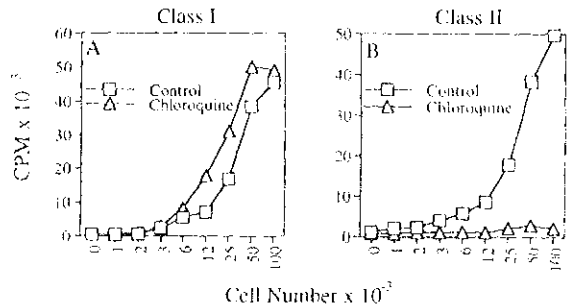

FIGURE 8. Effect of chloroquine on exogenous OVA presentation on MHC class I and class II molecules. DC2.4 cells (2×10^6/ml) were pretreated with 100 μM chloroquine for 30 min (open triangle) or with medium alone (open square) followed by the addition of bead-conjugated OVA (50 μg/ml), further incubated for 6 h, and fixed with 1% paraformaldehyde. Cells were then titrated in microtiter plates (100 μl), and the T-T hybrids (5×10^4/well), RF33.70 (*A*) and MF2.2D9 (*B*), were added to the cultures (200 μl total) and handled as described in *Materials and Methods*.

Ag-presenting capability was similar to that of A3.1A7, a macrophage cell line (Fig. 6*B*) (14). Since the DC were pure clones, we conclude that the pathway for presenting exogenous Ag on class I molecules is active in these APCs.

We also examined the ability of the DC to present Ag on MHC class II molecules. These cells presented soluble OVA to an OVA plus IA^b-specific T-T hybrid (Fig. 6*D*). Similar to the class I pathway, DC2.4 presented bead-bound OVA more efficiently than soluble OVA. Therefore, a DC can acquire exogenous Ag and present it simultaneously on both class I and class II molecules. Compared with similarly derived macrophage cell lines, the DC expressed higher levels of MHC class II molecules and were more potent at class II Ag presentation (data not shown). Without IFN-γ stimulation, the macrophage cell lines were either incapable or only weakly able to present Ag on class II (14).

Presentation of particulate OVA was inhibited by cytochalasin B

Phagocytosis was previously shown to be crucial for the presentation of particulate OVA by macrophages (11, 14). Here we examined the effect of cytochalasin B, an inhibitor of phagocytosis, on the presentation of OVA by DC. Treatment with cytochalasin B (5 μg/ml) inhibited the presentation of bead-bound OVA on MHC class I molecules (Fig. 7*A*). In contrast, this agent did not affect presentation when OVA was loaded directly into the cytosol (Fig. 7*B*), which indicates that it is interfering with an early event in the exogenous pathway and not at other steps in the class I pathway.

Presentation by DC of exogenous Ag on MHC class I molecules is chloroquine insensitive

The presentation of exogenous Ags on MHC class I molecules in some cases involves proteolysis of the Ag in the cytosol (8–10), while in other cases the proteolysis appears to occur in the endocytic compartment (7, 11–13). It was of interest to determine which of these mechanisms was operative in DC. To investigate whether presentation required proteolysis in acidic vesicles, we treated the DC with chloroquine during exposure to exogenous Ag. Chloroquine raises the pH in the endosomal and lysosomal compartments and thereby inhibits protein hydrolysis by cathepsins, which require an acidic environment for activity (50, 51). Treatment with chloroquine did not inhibit the presentation of particulate OVA on MHC class I molecules (Fig. 8*A*). In fact, this agent actually slightly enhanced this presentation, suggesting that proteolysis in vesicles might be limiting the availability of Ag for class I presentation. In contrast, chloroquine treatment completely inhibited the presentation of bead-bound OVA on class II molecules, as expected (Fig. 8*B*) (52). This latter finding serves as a positive control for the effectiveness of chloroquine in blocking proteolysis in vesicles. These results suggest that the class I-presented peptides are being generated outside of the endocytic compartments in DC.

Role of the proteosome in class I presentation by DC

The other major pathway for degrading proteins in cells is mediated by proteasome in the cytosol and nucleus (53). This pathway is responsible for generating the majority of class I presented peptides from endogenous cellular and viral proteins (2) and has been implicated in the presentation of exogenous Ags by macrophages (9). Therefore, we next examined the effects of peptide aldehyde inhibitors of proteasome (2) on the presentation of exogenous OVA by DC. As shown in Figure 9*A*, two of these inhibitors, LLnL and MG132, inhibited the presentation of particulate OVA on MHC class I molecules. In contrast, a closely related peptide aldehyde, LLM, did not inhibit the presentation. This agent serves as a specificity control because it has activity similar to those of LLnL and MG132 on thiol proteases, but is much less potent against the proteasome (2). Furthermore, LLnL and MG132 did not inhibit the presentation of the OVA peptide SIINFEKL expressed in cytosol from a minigene in a vaccinia virus construct (40) (Fig. 9*B*). These results indicate that LLnL and MG132 inhibit the presentation of exogenous OVA by inhibiting peptide generation by the proteasome and not by affecting other steps in the class I pathway.

Brefeldin A inhibited bead-conjugated OVA on both MHC class I and class II molecules

Brefeldin A blocks the exocytosis of proteins from the endoplasmic reticulum and Golgi complex and thereby prevents newly assembled peptide-MHC molecules from reaching the cell surface (54). The presentation of exogenous Ag on MHC class I molecules by the vacuolar pathway has been reported to be insensitive to this inhibitor (7, 11, 13), while presentation by the cytosolic pathway is inhibited by brefeldin A (8–10). We therefore examined the

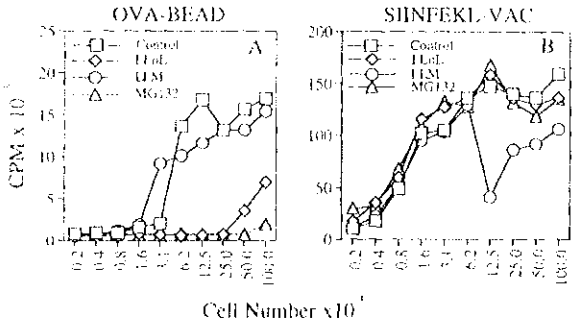

FIGURE 9. Exogenous OVA presentation was inhibited by proteasome inhibitors. DC2.4 cells were preincubated with medium alone (open square), 40 μM LLnL (open diamond), 40 μM LLM (open circle), or 10 μM MG132 (open triangle) for 30 min followed by the addition of bead-conjugated OVA (50 μg/ml) for 6 h (*A*) or infection with recombinant vaccinia virus encoding the SIINFEKL peptide (10 plaque-forming units/cell) for 3 h (*B*). Cells were then fixed with 1% paraformaldehyde and incubated with RF33.70 (10^5/well) for 20 h (200 μl). Cultures were then handled as described in *Materials and Methods*.

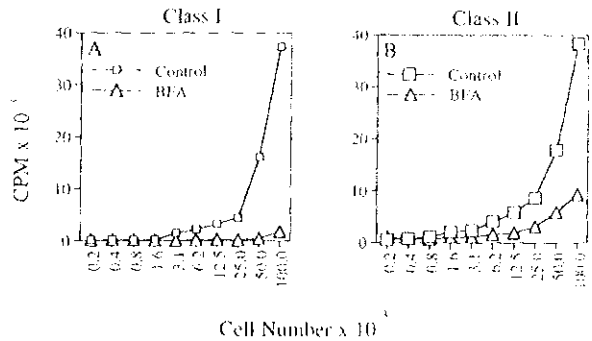

FIGURE 10. Effect of brefeldin A on exogenous OVA presentation on MHC class I and class II molecules. DC2.4 cells (2×10^6/ml) were pretreated with 5 μg/ml brefeldin A for 30 min (open triangle) or medium alone (open square) followed by addition of beads conjugated OVA (50 μg/ml), further incubated for 6 h, and fixed with 1% paraformaldehyde. Cells were then titrated in microtiter plates (100 μl), and the T-T hybrids (5×10^4/well) RF33.70 (*A*) and MF2.2D9 (*B*) were added to the cultures (200 μl total) and handled as described in *Materials and Methods*.

effect of this agent on the presentation of exogenous OVA by DC. Brefeldin A (5 μg/ml) completely inhibited the presentation by DC2.4 cells of particulate OVA on MHC class I molecules (Fig. 10*A*) and also inhibited the presentation of OVA on MHC class II molecules (Fig. 10*B*), suggesting a role for newly synthesized class II molecules in presentation of Ag by these DC.

Discussion

Properties of cloned DC

DC are present in both lymphoid and somatic tissues, but in relatively low abundance (55). Consequently, it is difficult to obtain large numbers of these cells with a high degree of purity. Many types of studies would be facilitated by the availability of reliable methods to isolate large numbers of homogeneous DC. Larger numbers of these cells can be obtained by culturing precursors from peripheral blood or bone marrow in GM-CSF and other cytokines (44, 45). This has been an important advance; however, these cultures typically contain other contaminating cell types, and this approach has not allowed the isolation of cloned DC lines. We also failed to grow clones of DC from bone marrow cultures transduced with GM-CSF. Presumably, GM-CSF stimulation is not sufficient for immortal growth of DC or their progenitors. This is consistent with previous studies examining the reconstitution of lethally irradiated mice with bone marrow that had been infected with a replication defective, retrovirus-expressing GM-CSF (56). Although a myeloproliferative disease occurred secondary to constitutive GM-CSF secretion in the primary recipients, this did not evolve into a clonal leukemia. Moreover, the myeloproliferative disorder could not be transplanted into secondary recipients (56). However, we found that supertransfection of *myc* and *raf* into GM-CSF-transduced cultures immortalizes these cells. In this case GM-CSF probably acts in a paracrine manner to expand infectable DC because immortal growth is maintained without the clones producing detectable levels of this cytokine. Similar approaches to immortalize DC using oncogenes (without GM-CSF) have been reported by others (57, 58), although whether the isolated cells are bona fide DC has not been firmly established in all cases.

The initial criteria we employed for identifying DC was morphologic. These clones had very obvious dendritic processes and veils, which was an appearance we had never observed in cells growing from fresh bone marrow infected with the same *myc*- and *raf*-expressing retrovirus (a procedure yielding macrophage and B cell clones) (14) (our unpublished observations). That at least some of these cells were indeed DC was confirmed by their expression of specific DC markers, DEC-205 and 33D1. These cells also expressed other molecules that are typical for DC, including high levels of B7 family members and MHC class I and class II molecules. They also expressed other receptors that are not present on freshly isolated DC, such as CD32 and Mac1, but which are found on cultured DC (47, 57, 59, 60).

The DC clones can avidly internalize micrometer-sized particles. This process requires microfiliments, and the particles are found in cells in large vacuoles. Therefore, the DC are phagocytic. This property has previously been observed for cultured DC and DC resident in tissues (61, 62). We show that this activity is important for the presentation on MHC molecules of peptides from particulate Ags (further discussed below).

We also isolated clones with similar morphology but that lacked expression of DEC-205 and 33D1. Given the similarity of their appearance and their expression of other cell surface molecules to the DEC-205-positive clones, we favor the possibility that these clones represent different subsets or different stages of maturation of DC. This interpretation would be consistent with other data demonstrating phenotypic heterogeneity in freshly isolated and cultured DC (63–65). However, in the absence of other objective criteria, it is difficult to rule out the possibility that these DEC-205-negative cells represent some other unrelated cell lineage or that their phenotype is abberent and an artifact of the immortalization conditions.

The immortalized DC lines are homogeneous and easily grown. These properties should be useful to studies exploring the cell biology and biochemistry of these cells. We have used them to analyze the Ag-presenting pathways that are operative in these cells.

Presentation of exogenous Ags by DC

The major question addressed by the present study is whether DC are capable of presenting Ags from the extracellular fluids on MHC class I molecules. Previously, cells with this capability were detected in fractions from both spleen and thymus enriched in DC

(5, 16). However, these same fractions contained some macrophages (5), and macrophages have been shown to have this Ag-presenting activity (11, 14). Our present results conclusively demonstrate that cloned DC that are free of macrophage contamination can present exogenous Ags on class I molecules. This pathway of presentation was detected in multiple different clones.

Soluble OVA added to culture medium was presented on class I molecules by DC, but required high concentrations of Ag. Approximately 1000-fold less Ag was required when the OVA was conjugated to a microsized particle that was internalized by phagocytosis. This enhanced presentation was blocked by cytochalasin B, which disrupts phagocytosis but not other endocytic processes. These results are similar to earlier findings with macrophages (14) and indicate that class I molecules can monitor the contents of phagosomes and potentially other endocytic compartments in DC. One such compartment might be the macropinosome, because this is a site in other cells that has been implicated in the delivery of exogenous proteins into the class I pathway (8), and macropinocytosis occurs in DC (66).

Two distinct pathways have been described for the presentation of internalized Ags on class I molecules of macrophages. One is independent of the proteasome and TAP and is resistant to brefeldin A (7, 11–13). In this case it is thought that the presented peptides are generated in the endocytic compartment and then bind to class I molecules on the plasma membrane. The other pathway requires proteasome and TAP and is sensitive to brefeldin A (8–10). In this case, Ags are transferred from phagosomes into the cytosol where they follow a common final pathway with endogenous Ags. The presentation of OVA particles by DC is sensitive to inhibitors of the proteasome and brefeldin A, but resistant to chloroquine. Therefore, it appears that in these APCs OVA is following the phagosome to cytosol pathway. Whether DC also have a vacuolar pathway for class I presentation remains to be determined.

Compared with macrophage lines, the cloned DC present exogenous Ag with similar efficiency to MHC class I-restricted T-T hybridomas. Nevertheless, it is likely that these cells will be more effective in stimulating responses to these Ags because of their potent immunostimulatory properties. T-T hybrids only require stimulation through their TCR, while normal T cells require additional signals, such as B7-1 and B7-2, which are expressed at high levels on DC. Moreover, the DC clones are constitutively better class II-presenting cells than macrophages, and this correlates with higher levels of expression of MHC class II molecules.

There is considerable evidence that the pathway for presenting exogenous Ags on class I molecules is active in vivo (6). Macrophages and DC isolated from immunized animals present the injected Ag on their class I molecules. Moreover, injection of antigenic particles primes CTL responses (6, 20). This pathway probably plays an important role in generating immunity in several pathologic situations, including responses against tissue transplants, tumors, and possibly even viruses (3, 17–19). In some of these situations, the exogenous pathway may be the major mechanism for initiating responses. This may be because the somatic cells that are producing the Ag are themselves poor stimulators of immunity, and professional APC, such as the DC, are needed to prime responses (15).

The exogenous class I pathway can potentially be exploited to generate CTL immunity to proteins in vaccines. Conventional vaccine preparations generally fail to elicit CTL responses, presumably because the Ags fail to be presented in vivo. However, particles can be used to target proteins into phagocytes, and this is effective in conferring protective CD8 T cell immunity (6, 20). The existence of the phagosome to cytosol pathway in DC makes this approach particularly attractive. Studying this pathway in the cloned DC should help to optimize approaches for targeting Ags into these key APCs.

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MATERIAL TRANSFER AGREEMENT

Dana-Farber Cancer Institute, Inc.

The University of Western Ontario at 1151 Richmond Street, London ON N6A 3K7 and its investigator(s) Dr Lakshman Gunaratnam (hereinafter collectively referred to as "Recipient"), in consideration of the receipt of biological materials (which material has been provided to Dr. Mansour Haeyrfar of The University of Western Ontario by way of Material Transfer Agreement dated September 29, 2006 and Dana-Farber Cancer Insitite (hereinafter "DFCI") herby consents to Dr. Haeyrfar providing said Material to Dr. Lakshman Gunaratnam) hereby agree to the following terms and conditions:

1. The biological materials to be provided to Recipient are: DC2.4. Material(s) shall mean the above referenced biological materials plus progeny, unmodified derivatives and any accompanying know-how or data.

2. The Materials shall be used exclusively for non-commercial research by Recipient to study Testing conditions that lead to DC maturation after renal transplantation.. The Material(s) shall be used solely by the named investigator and those under his or her direct supervision. Materials will not be used for *in vivo* testing in human subjects. Use will be in compliance with all applicable Federal, State and local laws and regulations, including, but not limited to animal welfare laws and regulations.

3. The Materials are the property of DFCI. Ownership of modifications and derivatives of Materials will be determined by the parties hereto depending upon (a) their relative contribution to the creation of said modifications and derivatives, which is to be considered but not required in said negotiation; and (b) any applicable laws and regulations relating to inventorship.

4. Recipient shall not sell or otherwise distribute Materials to a third party for any purpose. This Agreement and the resulting transfer of Material constitute a non-exclusive license to use the Material solely for the basic research or other not-for-profit purposes described herein. Recipient shall not use Materials for any products or processes for profit-making or commercial purposes.

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8. Recipient will arrange the return to DFCI or disposal of all unused Material whenever investigation for which it has been supplied discontinues or is terminated. In the event investigator(s) transfer to another institution, a new Material Transfer Agreement is to be executed.

9. The Material hereunder provided is experimental in nature, and it is provided WITHOUT ANY WARRANTIES, EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR USE. DFCI MAKES NO REPRESENTATION AND PROVIDES NO WARRANTY THAT THE USE OF THE MATERIAL

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Authorized

Institutional Officer: _____ Investigator: Dr Lakshman Gunaratnam

Title: _____ Title: _____

Signature: _____ Signature: _____

Date: _____ Date: _____

Acknowledgement: Dr. Mansour Haeyrfar, in consideration of section 1 as provided, hereby acknowledges and consents to the transfer of DC2.4 cells to Dr. Lakshman Gunaratnam.

By:_____

Approved by: DANA-FARBER CANCER INSTITUTE, INC.

_____ Date: _____

Anthony A. del Campo, MBA

Vice President, Office of Research and Technology Ventures

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THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: September 25, 2009
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biohazardous agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biohazards being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) for distribution to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety

PRINCIPAL INVESTIGATOR	LAKSHMAN GUNARATNAM
SIGNATURE	
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Location of experimental work to be carried out: Building(s) SDRI Room(s) 230, 229, 231

*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 12.0, Approvals).

FUNDING AGENCY/AGENCIES: KRESCENT / KFOC SUBMITTED, START-UP
GRANT TITLE(S): Mechanism of inhibition of danger signaling and renal transplant rejection by Kidney Injury Molecule-1

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED. A GRANT SUMMARY PAGE MAYBE ADEQUATE IF IT PROVIDES SUFFICIENT DETAIL ABOUT EACH BIOHAZARD USED.

Names of all personnel working under Principal Investigators supervision in this location:

Xizhong Xhang, MD, PhD
Ola Ismail, B.Sc.

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ⊘ YES O NO
(including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? O YES ⊘ NO
If YES, please give the name of the species. _____
What is the origin of the microorganism(s)? _____
Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____
_____
_____

1.2 Please complete the table below:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
E. coli	O Yes ⊘ No	O Yes ⊘ No	O Yes ⊘ No	2 L	Invitrogen Commercial	⊘ 1 O 2 O 3
Lentivirus	⊘ Yes O No	⊘ Yes O No	O Yes ⊘ No	10^6 - Ifv	Commercial (SCAST)	O 1 ⊘ 2 O 3 +
	O Yes O No	O Yes O No	O Yes O No	10		O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ⊘ YES O NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	O Yes ⊘ No		Not applicable
Rodent	⊘ Yes O No	Mouse kidney, spleen, blood, bone marrow	2010-037 Pending
Non-human primate	O Yes ⊘ No		
Other (specify)	O Yes ⊘ No		

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Supplier / Source
Human	⊘ Yes O No	786-O, 769-P, HEK293 (HKL)	ATCC
Rodent	⊘ Yes O No	293T, in IMCD-3, JAWS II CMT-93, RAW, WT-9.	ATCC
Non-human primate	O Yes O No		
Other (specify) Porcine	⊘ Yes O No	LLC-PK1,	ATCC

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

* Please see attached sheet. for details

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required O 1 O 2 O 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? O YES ⊘ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		O Yes O No O Unknown		O 1 O 2 O 3
Human Blood (fraction) or other Body Fluid		O Yes O No O Unknown		O 1 O 2 O 3
Human Organs or Tissues (unpreserved)		O Yes O No O Unknown		O 1 O 2 O 3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ⊘ YES O NO If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ⊘ YES, complete table below O NO

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results
(DH5 α) E. Coli	pCDNA3	Commercial	KIM-1	Unknown oncogenic Induces phagocytosis of apoptotic cells

* Please attach a Material Data Sheet or equivalent if available.

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

4.3 Will genetic modification(s) involving viral vectors be made? O YES, complete table below O NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results
Lentivirus	pLVX-Puro Proprietary	Commercial Invitrogen/Clontech Santa Cruz Biotech	Kim-1 or shRNA/siRNA	See previous Section 4.2

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV ✓ YES, please specify see attached data sheets O NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ ✓ NO
- SV 40 Large T antigen ✓ YES O NO
- E1A oncogene ✓ YES O NO
- Known oncogenes O YES, please specify _____ ✓ NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ ✓ NO

JQ

4.5 Will virus be replication defective? ✓ YES O NO

4.6 Will virus be infectious to humans or animals? ✓ YES O NO

4.7 Will this be expected to increase the containment level required? O YES ✓ NO II+

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES ✓ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

5.2 If YES, please specify which biological agent will be used: _____
Please attach a full description of the biological agent.

5.2 Will the biological agent be able to replicate in the host? O YES O NO

5.3 How will the biological agent be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted:_____

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? ✓ YES ~~✓ NO~~ If no, please proceed to section 7.0

6.2 Name of animal species to be used Musculus

6.3 AUS protocol # 2010-037 - Pending

6.4 Will any of the agents listed in section 4.0 be used in live animals O YES, specify: _____ ✓ NO

6.5 Will the agent(s) be shed by the animal: O YES O NO, please justify:
N/A

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

- Pound source dogs O YES ~~O NO~~
- Pound source cats O YES ~~O NO~~
- Cattle, sheep or goats O YES ~~O NO~~
- Non-human primates O YES, please specify species _____ ~~O NO~~
- Wild caught animals O YES, please specify species & colony # _____ ~~O NO~~
- Birds O YES ~~O NO~~
- Others (wild or domestic) O YES, please specify _____ ~~O NO~~

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? ~~O YES~~ O NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s) C3 Exotoxin, : Phalloidin
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin C3 50ng (mice) / Phalloidin 2.5mg/kg (mice)

8.4 How much of the toxin is handled at one time*? C3 10-20ng / Phalloidin (7 μmoles)

8.5 How much of the toxin is stored*? C3 25 μg / Phalloidin (500 mL x 14 μM)

8.6 Will any biological toxins be used in live animals? O YES, Please provide detais:_____ ~~O NO~~

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

9.0 Insects Requiring CFIA Permits

9.1 Do you use insects that require a permit from the CFIA? O YES ~~O NO~~
If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the life stage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-time" use, give location: _____

9.6 Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

9.7 Please attach the CFIA permit.

9.8 Please describe any CFIA permit conditions:

_____
_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

10.0 Plants Requiring CFIA Permits

10.1 Do you use plants that require a permit from the CFIA? O YES ✓ NO
If no, please proceed to Section 11.0

10.2 If YES, please give the name of the species. _____

10.3 What is the origin of the plant? _____

10.4 What is the form of the plant (seed, seedling, plant, tree...)? _____

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____
_____

10.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:
_____
_____
_____

10.8 Is the CFIA permit attached? O YES O NO
If NO, please forward the permit to he Biosafety Officer when available.

10.9 Please describe any CFIA permit conditions:
_____
_____
_____

11.0 Import Requirements

11.1 Will any of the above agents be imported? O YES, please give country of origin_____
If no, please proceed to Section 12.0 ✓ NO

11.2 Has an Import Permit been obtained from HC for human pathogens? O YES O NO

11.3 Has an import permit been obtained from CFIA for animal or plant pathogens? O YES O NO

11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE  _____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

13.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 O 2+ O 3
SDRI 231A

13.2 Has the facility been certified by OHS for this level of containment?
O YES, permit # if on-campus ~~ee~~ inspected June 29, 2010 dl
O NO, please certify
O NOT REQUIRED for Level 1 containment

14.0 Procedures to be Followed

14.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca/

SIGNATURE _____ Date: 04/19/2010

14.2 Please describe additional risk reduction measures will be taken beyond containment level 1, 2, or 3 measures, that are unique to this agent.
-

14.3 Please outline what will be done if there is an exposure to the biohazards listed, such as a needlestick injury:

First Aid → Cleanse wound → Emergency room visit → Report to occupational health

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: G.M. Kidder
Date: 29 June 2010

Safety Officer for Institution where experiments will take place: SIGNATURE: J Stanley
Date: June 29, 2010

Safety Officer for University of Western Ontario (if different from above): SIGNATURE: _____
Date: _____

Approval Number: BIO-UWO-0256 Expiry Date (3 years from Approval): June 28 2013

Special Conditions of Approval:

Follow SOP attached. - Protocol... Lentiviruses.

Adhere to maximum amounts to handle / store toxins (per attached e-mail).

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

Subject: Re: Biohazardous Agents Registry Form: Gunaratnam
From: Lakshman Gunaratnam <Lakshman.Gunaratnam@lhsc.on.ca>
Date: Thu, 03 Jun 2010 14:46:33 -0400
To: jstanle2@uwo.ca

* new info June 3, 2010

Sorry
O.1ug (70nM x 1/1000L x 1490 MW) max per use.
lakshman

Lakshman Gunaratnam, MD.,M.Sc.
Assistant Professor of Medicine
Nephrologist, University Hospital
London Health Sciences Centre
Schulich School of Medicine and Dentistry
University of Western Ontario
Siebens Drake Research Institute
1400 Western Road, Room 230B,
London, ON N6G 2V4

Phone: (519) 661-2111, Ext.89120
Email: gunaratl@lhsc.on.ca

> Jennifer Stanley <jstanle2@uwo.ca> 06/03/10 1:52 PM >>>

Thanks Dr. Gunaratham

Can you tell me the maximum amount of phalloidin handled at one time - please provide the amount in ug or mg

I think you missed this question?

Jennifer

On 6/3/2010 1:48 PM, Lakshman Gunaratnam wrote:

> Please see below for confirmation:
>
> 1. Please confirm the following for the exotoxin:
> LD50 - 50 ng/kg (for mice)
> max amount handled at one time - 50-100 ng
> max amount stored - 20 ug (0.1mg/ml x 0.2ml)
>
> 2. Please let me know the following for the phalloidin:
> LD50 - 2 mg/kg (for mice)
> max amount handled at one time - please provide the amount in ug or mg
> max amount stored - please provide the amount in ug or mg = 10ug (14uM stock x 1490 MW x 0.5ml)
>
> Thank you.
>
> Lakshman Gunaratnam, MD.,M.Sc.
> Assistant Professor of Medicine
> Nephrologist, University Hospital
> London Health Sciences Centre
> Schulich School of Medicine and Dentistry
> University of Western Ontario
> Siebens Drake Research Institute
> 1400 Western Road, Room 230B,
> London, ON N6G 2V4

0.1ug (see above)

new info
June 10/10

- DC.4 cells
- They are transformed (SV40 T antigen) mouse dedritic cells. They were made by Dr. Ken Rock while at Dana Farber. Sorry for the oversight.

1. Please confirm the cell lines that you use (it was difficult to decipher the cell lines in Table 2.3)

- MDCK
- LLC-PK1
- RAW
- HEK293, HEK293T, 293T
- Jurkat
- JAWSII
- HK-2
- WT-9 DC4
- mIMCD-3
- CMT-93
- 769-P and 786-O renal cell cancer ell lines
- We will use/establish stably transfected cells expressing KIM-1 or KIM-1-GFP

Mouse Primary Cells

- Renal tubular epithelial cells from kidney
- Dendritic cells from bone marrow, lymph nodes or spleen
- CD4 and CD8 T cells from lymph nodes or spleen or kidney
- Splenocytes
- Thymocytes

2. Please send some information on the lentiviral vectors, etc. - such as an MSDS and/or website information (see attaches printouts)
- see attached printouts

3. Please send a description of the work you do that describes how the biohazards are used, stored and disposed of. Be sure to describe the modifications you do with the lentiviral vectors.

DMSO: Will be used as a preservative to cryopreserve cells. DMSO will be used at 10% in fetal bovine serum. DMSO accumulates in the medium at <1% concentration and will be discarded in the sink with refuse media after addition of bleach. DMSO will be stored in the stored in the contained provided by the manufacturer and stored as described in MSDS (provided).

C3 Exotoxin: C3 is used to block RhoA activity in cells in culture at 1microgram/mL. Media containing C3 will be discarded as stated above (DMSO) given that C3 at these diluted concentrations is not harmful to the environment. C3 is stored at -20 degrees Celsius as indicated by MSDS.

E.coli: Will be used for cloning and plasmid preparation. Standard precautions will be used under biohazard safety level 1.

Lentivirus: Lentivirus technology will be used to introduce or silence (SiRNA/shRNA) our gene of interest, KIM-1, into established cell lines or primary cells in culture. We are planning on purchasing Lentivirus for SiRNA/shRNA from Santa-Cruz Biotechnology (http://datasheets.scbt.com/sc-61691-sh.pdf). Expression vectors will be purchased from Clonetech (http://tools.invitrogen.com/content/sfs/manuals/virapower_lentiviral_system_man.pdf).

As far as we know KIM-1 is not a proto-oncogene. We will follow all standard safety practices when handling or discarding lentiviral particles (described in attached document from Queen's University Environmental Health and Safety).

Questions raised in review of protocol:
1. Where will it be done?
All lentiviral work will be done in a designated bio-safety cabinet in a closed room. All personnel will wear N95 masks at all times (during interaction) and use double-glove technique. All contaminated equipment will be placed in bleach solution as described in
2. Which cells (specifically) do you intend to infect with lentiviral particles?
Lentiviral technology will be used to express KIM-1 or SiRNA/shRNA targeting KIM-1 in mouse primary cells and cell lines that are not easily transfectable at high efficiency. The cell types to be infected with lentivirus are: Primary mouse kidney tubular epithelial cells, mouse primary T cells, HEK-293 (ATCC), LLC-PK1 (ATCC), 769-P(ATCC), 786-O (ATCC), CMT-93 (ATCC).

Describe your overall experimental design - a grant summary may suffice. Please see attached summary from recently submitted grant/award. The primary purpose of the lentiviral expression system is to decipher how the presence or absence of KIM-1 in our cells affects phagocytosis of apoptotic/necrotic cells and how this affects downstream signaling events that can lead to inflammation at the organism level (i.e. in transplantation). We do not plan to use lentivirus in animals.

We will need and adhere to biohazard containment level 2 precautions in conducting all this work (http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm) and http://www.clontech.com/products/detail.asp?product_id=172594&tabno=2 (Clonetech).

Prepared by
Lakshman Gunaratnam, MD

Western

Protocol for Handling Recombinant Replication-deficient Lentiviruses

Lentiviral vectors are different from the commonly used adenovirus-based gene delivery systems because the gene of interest becomes stably integrated into the host cell's genome. The efficiency of lentiviral systems is impart due to the fact that they are actively imported into the nuclei of dividing, as well as non-dividing cells, as opposed to traditional retroviruses.

The lentiviral genome contains nine genes but only three of those are required to package a replication-deficient virus. The three essential genes are Gag, Pol and Env and they can all be provided in trans. Gag encodes a capsid proteins and Pol is required for the viralreverse transcriptase, RNase, protease and integrating functions. The Env, or, envelope gene encodes a transmembrane glycoprotein that also determines the tropism of the viral particle (ie. the specificity of the virus for a particular host cell). **In the ViraSafe Ecotropic Packaging system from Cell Biolabs, Inc. the *env* gene encodes a glycoprotein from murine ecotropic retrovirus, thus providing a viral particle that can transduce only mouse and rat cells with high efficiency**. However, other types of retroviral envelope genes can be used. If the *env* gene is from a xenotropic retrovirus the lentivirus vector will only infect non-mouse cells. If *env* gene is from an amphotropic retrovirus then it will infect cells in a species independent fashion. More frequently though the envelope gene G from Vesicular Stomatitis Virus (VSV) is used to pseudotype the lentivirus core with an envelope protein that provides a broader host range with better infectivity in some cases. The actual lentiviral genome that gets packaged is devoid of any coding sequence and the U3 region of the genomic RNA is deleted so that when the RNA genome is reverse transcribed it generates a self-inactivating DNA version of the RNA genome that after integration cannot lead to the generation of viral progeny. The remainder of the viral genome (*ie.* cis-elements only) is used to construct and direct the packaging of different lentiviral cloning vectors when the cloning vectors along with plasmids that provide the gag, pol and env genes in trans are co-transfected into packaging cell lines (usually HEK 293T cells). Cells containing and expressing all the necessary components then produce new infectious replication-defective viral progeny over the next 24 to 72 hrs.

Note! Only laboratory personnel that have been informed about safety precautions and working routines, and have permission from the person in charge are allowed to enter the laboratory during Lentiviral work production. <u>This also includes cleaners and service-personnel</u>.

Principle:

All procedures for handling or manipulating Lentivirus should be carried out at Biosafety Level 2 (BL2) with the use of Containment Level 3 operational practices. All work will be done in a biological safety cabinet (BSC) by authorized personnel wearing gloves, safety glasses, shoe covers, and overgowns that cover the front and close at the back. Personal items (eg. purses) will not be brought into the containment room. All protective clothing will be removed upon completion of the work and left in the room or disposed of as waste (shoe covers, gloves). Protective items to be re-used will be autoclaved. Overgowns will be kept on a coat rack within the containment room. No work with these viral vectors is permitted on the open bench. The door to the laboratory must remain closed

Working precautions for handling Lentivirus:

1. All experimental materials shall be handled with care.
2. The door to the containment room shall remain locked.
3. Within the BSC:
   a. For small quantities of low (cell lysate) and high (purified) titer Lentivirus, use sterile, aerosol barrier-containing pipette tips.
   b. For larger amounts (more than 1ml) of low titer lysates use sterile serological disposable pipettes.
   c. The maximum amount of infected growth media handled at one time should never exceed 500 mL.
4. Using a dunk tank, plastics will first be either filled (eg. pipette tips and serological pipettes) or rinsed (eg. plates and flasks) with Wescodyne Solution (20% Wescodyne/40% ethanol/40% water), drained, and then put into a high-density 4mil polyethylene plastic biohazard bag lined with a cardboard box prior to autoclaving.
5. Concentration of the viral particles will be done using either appropriate ultracentrifuge rotor or by using Amicon Ultra-15ml 100k MWCO centrifugal filter devices. All centrifugation shall be done in closed buckets with aerosol-tight lids. Loading and unloading of samples into the sealed buckets will be done in the BSC. The sealed centrifuge buckets will be sprayed with 70% ethanol before removing from BSC.
6. Sharps shall be eliminated from experimental procedures to prevent injuries. No needles or Pasteur pipettes will be used in the production and use of lentivirus.
7. Double gloves shall be worn at all times when working with viral vectors. Gloves will be sprayed with 70% ethanol and then the outer glove removed inside the hood by using the inside-out technique before disposing into biohazard waste dunk tank located inside the BSC. Remove lab coat and boots and dispose of in biohazard waste. Spray inside glove with 70% ethanol and remove using the inside-out technique, dispose into biohazard waste bag. Wash hands immediately after removing gloves and before leaving work area. Never wear gloves outside of the laboratory, or touch things with gloved hands.

8. During any lentiviral work, signs and labels shall be placed to indicate each area where viral vectors are used and stored (BSC, incubators, freezer, laboratory entrance doors, etc.)

Decontamination and disposal procedures:

All materials that come in contact with viral particles must be properly decontaminated prior to disposal. This requires that all material must be autoclaved prior to leaving the level 2 plus 3 room where the work is being done. This requires that the level 2plus 3 room must have an autoclave in it. Alternatively a portable autoclave may be used.

1. ***Disposal/decontamination of solid waste such as, paper tissues, pipette tips, etc.***: All solid waste (including disposable plastic wares) should be discarded in biohazard bags for the appropriate treatment (autoclaving) according to PHAC/CFIA guidelines, institutional practices and guidelines prior to disposal. Information on hazardous waste disposal is found in the Hazardous Materials Management Handbook:
   http://www.uwo.ca/humanresources/docandform/docs/ohs1/manuals/hazardous_handbook.pdf
   Personnel must take the Biosafety training courses from Occupational Health and Safety and General Laboratory Safety and Hazardous Waste Management.
2. ***Disposal/decontamination of liquid waste***: All liquid materials (Lentivirus-containing media, buffers, washes) should be decontaminated inside safety cabinet by addition of wescodyne or Quatricide PV Solution prior to autoclaving.
3. ***Work surfaces inside cabinets*** should be decontaminated with Quatricide PV Solution allowing for a wet contact time of at least ?? minutes to ensure virus inactivation, followed by 70% ethanol again with at least ?? minutes of contact time.
4. ***Instruments, equipment*** and any other items that are not disposable and contact Lentivirus will be decontaminated with Quatricide PV Solution (again observe minimum contact time) and/or autoclaved.
5. ***Routine laboratory cleaning*** will be done by lab personnel within the containment room.

Accidents:

Spills:

Effective disinfectants (10% bleach, Wescodyne or Quatricide PV Solution) will be made available in the laboratory at all times and for immediate use. In the event of a spill or container breakage resulting in the unintentional release of a biological agent:

(i) Place bleach soaked paper towel or absorbent on the liquid
(ii) Pour a strong disinfectant solution (i.e. use same product used to soak the paper towel in (i), do not mix different agents together) around, but not on the spill, and mix the disinfectant with the spilled material cautiously;
(iii) Evacuate the laboratory for a time expected to be sufficient for decontamination of the mixed material, normally 20 minutes;

(iv) Carefully place absorbent paper into a bag for incineration;
(v) Decontaminate all surfaces exposed to the spill with the appropriate disinfectant allowing for appropriate contact times.

If aerosols may have been created in the spill or unintentional release, evacuate the laboratory for a time sufficient for most aerosols to settle, be dispersed, or removed by the ventilation system, usually 20-30 minutes. The use of respiratory protection should be considered for re-entry. Then proceed with items (i)-(v) above. During an emergency, the first priority is the protection of the health and safety of personnel, followed by the environment (i.e. sewer drains), followed by equipment or property.

Spills within a biological safety cabinet

- Leave the ventilation on
- All items within the cabinet should be disinfected (Walls and surfaces wiped down, equipment wiped down and/or autoclaved)
- Cover the spill area with paper towels or absorbent material
- Pour a strong disinfectant solution (i.e. use same product used to soak the paper towel in (i), do not mix different agents together) around, but not on the spill, and mix the disinfectant with the spilled material cautiously; Leave on for 20 to 30 minutes
- Pick up with absorbent material and place in biohazard bag to be then autoclaved
- Ventilation should run 10-15 minutes before continuing work in BSC

Spills within an incubator

- All shelves and walls within the incubator should be disinfected (walls and surfaces wiped down, and/or autoclaved)
- Cover the spill area with paper towels or absorbent material
- Soak the spill area with an appropriate disinfectant (i.e.Wescodyne, or Quatricide PV Solution) Pour the disinfectant from the outside surface of the absorbent material towards the inside, surrounding the spill. Leave on for 20 to 30 minutes (close the door of the incubator during the disinfection time)
- Pick up with absorbent material and place in biohazard bag to be then autoclaved
- Finish by wiping the incubator with 70% ethanol

First Aid:

In the case of any incident or accident, personnel must seek medical treatment and notify the Principle Investigator or Laboratory Supervisor. An accident/incident reporting form must be completed:
http://www.uwo.ca/humanresources/facultystaff/h_and_s/acc_inc/accident_inc_index.htm

Eye exposure from splash or aerosol:

Rinse a minimum of 15 minutes in eye wash or flush with water. Notify the Principal Investigator or Laboratory Supervisor, who will immediately contact Workplace Health at 519-661-2047 and direct the exposed employee to appropriate medical treatment and to report the incident.

Skin Exposure or Abrasions:

Contaminated skin or abrasions should be scrubbed with germicidal soap and copious amounts of water. Notify the Principal Investigator or Laboratory Supervisor, who will immediately contact Workplace Health at 519-661-2047 and direct the exposed employee to appropriate medical treatment and to report the incident.

Inhalation:

Remove person to fresh air. Notify the Principal Investigator or Laboratory Supervisor, who will immediately contact Workplace Health at 519-661-2047 and direct the exposed employee to appropriate medical treatment and to report the incident.

Gunaratnam.

------- Original Message --------
Subject: Re: Containment Level - Lentiviral project
Date: Fri, 23 Apr 2010 09:35:57 -0400
From: Permit-Permis <permitpermis@phac-aspc.gc.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>

Dear Jennifer Stanley
Thank you for contacting our Directorate with your questions.
I regret that the Office of Laboratory Security has a policy of not undertaking risk assessments of the human pathogens in facility's inventories, as many of the factors that come into play are specific to a particular location and application. The determination of what risk group to which your pathogens belong is your responsibility.
As a first means of assisting you in determining your inventory's risk groups(s), you may want to consult the schedules for Risk Groups 2, 3, and 4 that are appended to The Human Pathogens and Toxins Act. Schedules 2 to 4 of the Act provide, respectively, non-exhaustive lists or examples of the kinds of human pathogens that are included in each of risk groups 2, 3, and 4. See link below:
http://www2.parl.gc.ca/HousePublications/Publication.aspx?Docid=4015133&file=4
If you possess human pathogens that are not included in these schedules, then you would have to determine the risk groups of those pathogens yourself. First, you could consult the definitions of the Risk Groups that are provided in section 3 of the Act. Further, there is greater detail on the criteria for determining the risk group of a pathogen in sections 2.1, 2.3 and 7.2 of the Laboratory Biosafety Guidelines . See attachment below:

For more information you can visit our website;
http://www.phac-aspc.gc.ca/ols-bsl/pathogen/index-eng.php

Regards

Josee Davies
Regulatory Technologist/ technologiste en réglementation
Pathogen Regulation Directorate (formerly Office of Laboratory Security) /
Direction de la réglementation des agents pathogènes (anciennement le Bureau de la sécurité des laboratoires)
Public Health Agency of Canada/ Agence de santé publique du Canada
100 ch. Colonnade Rd. AL: 6201A Ottawa, Ontario, Canada K1A 0K9
Tel: (613) 957-1779
Fax: (613)941-0596

Jennifer Stanley <jstanle2@uwo.ca> 2010-04-22 02:53 PM
To Permit-Permis permitpermis@phac-aspc.gc.ca cc
Subject Containment Level - Lentiviral project

Hello there,
Please let me know what containment level you suggest for the following project:

Lentiviral technology will be used to introduce or silence (SiRNA/shRNA) our gene of interest, KIM-1, into established or primary cells in culture. We are planning on purchasing Lentivirus for SiRNA/shRNA from Santa-Cruz Biotechnology: http://datasheets.scbt.com/sc-61691-sh.pdf
Expression vectors will be purchased from Clonetech:
http://tools.invitrogen.com/content/sfs/manuals/virapower_lentiviral_system_man.pdf

Lentivirus technology will be used to express KIM-1 or siRNA/shRNA targeting KIM-1 in mouse primary cells and cell lines that are not easily transfectable at high efficiency. The cell types to be infected with lentivirus are: primary mouse cells (kidney and T cells) and ATCC cell lines HEK 293, LLC-PK-1, 769-P, 786-0 and CMT-93.

Regards,

Jennifer

Name of Candidate

Lakshman Gunaratnam

Project Title: **Mechanism of Negative Regulation of Danger Signaling and Kidney Transplant Rejection by Kidney Injury Molecule-1**

Abstract

Overview

Kidney transplantation remains the treatment of choice for patients with end-stage renal disease. One of the major problems in transplantation is immunologic rejection of the graft. As T cells are both required and sufficient for rejection of allotransplants, transplantation immunology research has largely focussed on the adaptive immune system. An increasing body of evidence suggests a key role for the innate immune response in activating dendritic cells (DCs) that ultimately direct allogeneic T cells to mediate allograft rejection. Release of endogenous "danger" signals by damaged tissue or necrotic allograft cells due to ischemia reperfusion injury (IRI) is a powerful activation signal for DCs. Kidney injury molecule-1 (KIM-1) is a novel scavenger receptor that is highly upregulated in renal tubular epithelial cells (RTECs) and converts them into phagocytes for apoptotic and necrotic cells. We hypothesize that upregulation of KIM-1 allows RTECs to inhibit allograft rejection by negatively regulating activation of DCs by HMGB-1, a key "danger" signal released by RTECs following IRI. By uncovering how RTECs regulate innate immunity, we hope to identify therapeutic strategies to prevent transplant rejection and improve overall graft survival in transplant patients.

Background

DCs like other innate immune cells use pattern recognition receptors (PRRs) to recognize conserved molecules termed pathogen-associated molecular patterns (PAMPs) on bacteria and other evolutionarily distinct organisms. Ligation of PRRs (e.g. Toll-like receptors, TLRs) on innate immune cells by PAPMs triggers a cascade of signalling events that lead to production of pro-inflammatory cytokines, adhesion molecules, chemokines and antimicrobial peptides that allow them to function as a fist line of defence against pathogens. Alloreactive T cells can recognize intact donor MHC molecules on "passenger" (present within the organ before harvesting) antigen presenting cells (APCs) (*1*) or, indirectly, when recipient APCs capture, process and present donor MHC and non-MHC alloantigens to recipient T cells in a host-MHC restricted fashion (*2*).(*3*). Engagement of naïve T cells by APCs in the absence co-stimulatory signals results in T cell anergy or apoptosis. Maturation of resting DCs to express co-stimulatory molecules can be triggered by binding of pathogens (or PAMPs) to PRRs on their surface. One of the vexing questions in transplant immunology is how naïve T cells become activated in the absence of pathogenic stimuli (e.g. PAMPs), especially, given that transplant surgery for the most part is a sterile procedure.

Work done by Matzinger and others, suggests that PRRs can also be activated by host-derived "danger" or "alarm" signals sent by injured, damaged or necrotic cells (*4-5*). A number of endogenous "danger" or damage associated molecular patterns (DAMPs) have been identified including high-mobility group B-1 (HMGB1), heat shock protein 70 (hsp70), hyaluronan (HA) and S100s (*6*). HMGB1, is a ubiquitously expressed and highly conserved chromatin-associated protein is released extracellularly by necrotic cells, but not by apoptotic cells (*7*) (*8*). HMGB1 can also be secreted by activated macrophages, DCs and NK cells or non-immune cells in response certain inflammatory stimuli such as LPS, IFN-γ or interleukin-1 (IL-1) (*9*). Binding of extracellular HMGB1 to its receptor(s), the receptor for advanced glycation end products (RAGE) and members of TLR family (i.e. TLR-2, TLR-4, and TLR-9), triggers several signalling pathways in target cells including MAPK and nuclear factor-κB (NF-κB), which in DCs, can trigger upregulation of co-stimulatory molecules, enhance antigen presentation via MHC class II and secretion of pro-inflammatory cytokines (e.g. IL-12, IL-6, IL-1β, IL-8, TNF-α, and RANTES) (*9-10*).

RTECs are a major target of IRI and often undergo apoptosis or even necrosis as a result (*11-17*). The importance of IRI in the regulation of T cell-mediated allograft rejection is highlighted by the fact that "parking" cardiac allografts in immunodeficient hosts to allow them to heal from IRI prevented acute rejection when grafts were exposed to active alloreactive T cells later on (*18*). Recent evidence has now implicated HMGB1 as an early mediator of inflammation in hepatic IRI as administration of neutralizing antibody to HMGB1 significantly decreased liver injury (*19*). These and several other studies together suggest that danger signals from injured (IRI) allografts may play a central role in priming naïve alloreactive T cells and promote rejection by stimulating DCs (*20-21*).

KIM-1 is a novel scavenger receptor protein that is highly upregulated *in vivo* on the surface of proximal RETCs within hours of IRI or after renal transplantation (*22-25*). Recently a number of groups including Bonventre's, demonstrated that KIM-1 binds to phosphatidylserine (PS), an "eat-me" signal exposed on the surface of cells undergoing apoptosis and can mediate phagocytosis of apoptotic and necrotic cells (*26-28*). *In vivo*, the remnants of apoptotic and necrotic cells can be visualized within phagosomes of KIM-1-expressing RTECs in rodent models of AKI (*23, 27, 29*). The exact physiologic role of KIM-1 in RTECs *in vivo* remains unknown.

Preliminary Data

Mice engineered to express phagocytosis-defective mutant KIM-1 are more susceptible to IRI and accumulate apoptotic and necrotic cells within the injured tubular lumen of kidneys (Bonventre, personal communication). Data presented in figure 1 suggests that HMGB1 can be detected in the urine of mice at least within twelve hours after IRI but not in sham treated mice. As HMGB1 is released from necrotic cells, we exposed KIM-1 expressing LLC-PK1 cells in culture to necrotic cells and measured residual extracellular HMGB1 in the conditioned medium after 24 hours to test if KIM-1 regulates the availability of this extracellular danger signal (Figure 2). The conditioned medium from KIM-1 expressing cells had significantly less extracellular HMGB1 compared to non-KIM-1 expressing controls suggesting that KIM-1 may play a role in regulating secretion and or removal of extracellular HMBG1 (or necrotic cells).

Rationale and Hypothesis

KIM-1 is highly upregulated in RTECs during IRI and that it is a scavenger receptor capable of mediating phagocytosis of necrotic cells. Together with the preliminary data presented here, we propose that HIM-1 may regulate danger signalling following IRI in renal transplantation.

Hypothesis: KIM-1 negatively regulates allogeneic Tcell immunity to kidney allografts by sequestering HMGB1 and blocking danger signalling to DCs.

Specific Aims and Experimental Plan

Specific Aim 1: To determine the mechanism by which KIM-1 regulates extracellular HMGB1. ***Rationale:*** Elucidating the exact molecular mechanism of how KIM-1 regulates HMGB1 availability may help us design experiments to specifically block this pathway in animal models and moreover uncover strategies to enhance this mechanism in a clinical setting. It would be important to distinguish whether KIM-1 is preventing release of HMGB1 by RTECs exposed to necrotic cells or by actively removing it from the extracellular medium. We have thus designed experiments to test the following mechanisms: First goal: Determine whether KIM-1 binds directly to HMGB1 (primary sequestration) and degrades it via receptor-mediated endocytosis. *Second goal:* Determine if passive release of HMGB1 is inhibited by KIM-1-dependent phagocytosis of necrotic cells (secondary sequestration). Third goal: Determine if KIM-1 expression on RTECs blocks HMGB1 release (from RTECs) when they are exposed to necrotic cells.

Methodologies: In order to test our hypotheses, we will use a variety of RTEC types such as LLC-PK1 cells, MDCK cells as well as primary mouse RTECs and non-tubular HEK293 cells. The basic assay will consist of exposing RTECs to either to no treatment, live cells, necrotic cells or recombinant HMGB1 (added to medium after monolayer has formed rHMGB1), allowing time for uptake or release, and then measured extracellular HMGB1 in the conditioned medium by western blotting and/or ELISA. To test whether KIM-1 binds to HMGB1, we will immunoprecipitate either molecule after exposing KIM-1-expressing RTECs to rHMGB1 or necrotic cells and then detecting the interaction by Western blot. To determine if internalized HMGB1 is degraded by RTECs, we will simultaneously treat cells with proteasome or lysosomal inhibitors before adding necrotic cells and then detecting the interaction by Western blot. To determine if bound HMGB1 is degraded we will simultaneously treat cells with proteasome or lysosomal inhibitors in the above experiment. Necrotic cells will be generated by exposing either Jurkat cells (ATCC) or primary mouse splenocytes to multiple freeze-thaw cycles (*5*).

Specific Aim 2: To determine whether KIM-1-epressing RTECs inhibit danger singling by extracellular HMGB1 and inhibit activation of DCs.

Rationale: HMGB1 can function as an adjuvant to stimulate allogeneic T cell immunity by activating DCs (danger signalling) by inducing phenotypic maturation of DCs (*10, 30*). First goal: Determine how conditioned medium from KIM-1-expressing RTECs after exposure to necrotic cells or rHMGB1 affects DC activation *in vitro*. Second goal: Determine how KIM-1-expressing RTECs co-cultured with DCs exposed to necrotic cells or

rHMGB1 (simultaneously) affect DC activation *in vitro*. Third goal: Determine how DC activated as above (first and second goals) affect allogeneic T cell responses (direct allorecognition).

Methodologies: We will use primary mouse (SCID) bone marrow-derived DCs (*5*) or widely available DC lines (e.g. ATCC-CRL-11904). RTECs will be cultured in monolayers in transwell plates with DCs or conditioned medium will be transferred from RTECs after exposure to necrotic cells or rHMGB1. For T cell activation assays, DCs (from C57BL/6 vs. DBA/2 mice) will be treated with as above (first and second goals) and then co-cultured with primary CD4 T cells isolated from mice or allergenic T cell clones before measuring proliferation using ^{3}[H]-thymidine or CFSE dye assays as described (*10*). Quantitative RT- and ELISA will be used to measure release of proinflammatory cytokines such as IL-12, IL-6, IL-1alpha, IL-8, TNF-alpha, and RANTES. Phenotypic maturation of DCs (after treatment as above) will be measured by flow cytometric analysis for increased surface markers such as CD83, CD54, CD80, CD40, CD58, and MHC class II and decreased CD206 expression . Alternatively ELISPOT can be used to measure T cell reactivity (*31*).

Specific Aim 3: To test if targeted deletion of kim-1 in murine kidney allografts exacerbates danger signalling and promotes allograft rejection. *Rationale:* HMGB1 (Figure 1) and KIM-1(*29*) can be detected in the urine of rodents subjected to IRI. In vitro studies often oversimplify complex regulatory pathways and may not represent finding *in vivo*. This aim will test if KIM-1 negatively regulates allograft rejection *in vivo*. First goal: Monitor survival, renal function and urinary KIM-1/HMGB1. Second goal: Perform histological and functional analysis on kidney tissue, DCs and T cells.

Methodologies: C57BL/6, H2-2^{b} kidneys will be transplanted into recipient (DBA/2, H-2^{d}) mice as described (by Noris et al.) by Dr. Zhang (our core veterinary surgeon). Syngeneic transplants will be used to monitor for non-alloimmune effects on the graft while sham treated mice will serve as naïve controls. Parallel groups of experiments will be performed. Mice in one group will be sacrificed on days 1,3, 6 and 12 and kidney grafts will be divided and used to perform: histological analysis (tubular injury, tubulitits, leukocyte infiltration, atrophy, total tubulointerstitial injury score), measure urinary KIM-1/HMGB1, in situ hybridization (KIM-1), RT-PCR (TLR-2, TLR4, TLR9, RAGE, TNF-α, MIP-2, MCP-1, IL-1β, IL-6), immunostaining (macrophages, granulocytes, T cells, B cells, DCs [CD11b+ and CD11c+], HMGB1, KIM-1, MHC-II [H-2^{b} and H-2^{d}]). We will also examine sections for total CD8+, CD4+, CD4+FoxP3 T cells per field. In the second group we will monitor survival, and measure serum BUN, creatinine, urinary KIM-1/HMGB1 at on odd days for 2 weeks and then at 30 and 60 days. We will measure recipient T cell alloreactivity exposing recipient splenocytes to irradiated donor (H-2^{b}) or third-party (DBA/1, H-2^{q}) splenocytes or isolated DCs (bone marrow or from the allograft). Sham treated mice will serve as additional controls in all experiments.

Expected Results:

We predict that KIM-1 expressed on injured RTECs decreases available HMGB1 by removing necrotic cells from the extracellular milieu (indirect sequestration). RTECs expressing mutant KIM-1 that cannot bind phosphatidylserine (data not shown) should behave like non-KIM-1-expressing cells. Given that HMGB1 and necrotic cells can activate DCs (*3, 5*), we expect that transfer of conditioned medium from KIM-1-expressing RTECs will stimulate DCs to a much lesser degree than non-KIM-1-expressing cells. This effect should translate into reduced allostimulatory effect of DCs activated by conditioned medium from KIM-1-expressing RTECs. Unless, KIM-1-expressing RTECs have a direct dominant positive effect on DCs after exposure to HMGB1, simultaneous co-culture experiments (specific aim 1) are unlikely to yield any differences in DC activation regardless of KIM-1 status. Finally, we do expect that mice that receive KIM-1-deficient kidneys will have exhibit higher incidence of rejection. We also expect that there will be a detrimental effect from KIM-1 deficiency even in the syngeneic recipients.

Where Funding Will Be Applied For:

1. Canadian Institutes of Health Research: Operating Grant (09/2010 deadline)
2. Kidney Foundation of Canada: Biomedical Research Grants (15/10/2010 deadline)
3. Natural Sciences and Engineering Research Council: Discovery Grant (08/210 deadline)

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1. Purpose:

To describe the biological safety risks of working with lentiviral vectors and the engineering and operational practices that are approved for mitigating these risks. Note that these are minimum standards and that some experiments may require higher containment practices. This should be decided jointly by the Principal Investigator and the University Biohazard Committee.

2. Applicable Legislation, Standards, Guidelines:

Public Health Agency of Canada Laboratory Biosafety Guidelines, 3rd edition, 2004
Canadian Food Inspection Agency Veterinary Standards for Animal Facilities
National Institutes of Health, Recombinant DNA Advisory Committee, Guidance on Biosafety Considerations for Research with Lentiviral Vectors (March 2006)
http://www4.od.nih.gov/oba/rac/Guidance/LentiVirus_Containment/index.htm

3. Requirements:

No one is allowed to work with lentivirus without having prior training by the Principal Investigator who supervises their work, or their designated technical expert. The worker should demonstrate good microbiological and tissue culture technique and an understanding of this SOP prior to being permitted to work with lentivirus.

The use of or generation of any new lentiviral constructs must to be cleared with the Principal Investigator.

The use or generation of any new lentiviral construct that involves a different transgene or a different type of alteration than that already approved by the Biohazard Committee (e.g. the knock-out rather than the expression of a particular protein, its subunits or its mutants) must be communicated to the Biohazard Committee via an application for an amendment to the laboratory's Biohazard Permit. Constructs that involve different mutations in a particular protein, where the work on that protein has already been approved, do not require an amendment as long as the Principal Investigator is confident that no additional biohazard risk is being created.

4. Biological Safety Risk:

- Lentiviral particles are usually pseudotyped with the vesicular stomatitis virus glycoprotein (VSVG), an envelope protein which gives the virus the ability to infect many human and mammalian cell types. The skin affords some protection, but the virus may enter the body through chapped skin or wounds or by infecting mucosal surfaces (eyes, nose, mouth).

Pseudotyping with other proteins will change the tropism of the viral particle and therefore alter the risk.

- Lentivirus is an enveloped virus, so it is susceptible to inactivation by sufficiently long treatment with 70% ethanol or freshly diluted 10% bleach.
- The direct effect on the infected cell will depend in part on the protein that the virus is engineered to produce (the product of the transgene).
- Lentivirus can integrate into chromatin in non-dividing cells.
- Lentivirus integrates into chromatin (randomly) so there is an unpredictable risk associated with gene disruption. Note that some gene disruptions may promote abnormal cell growth.
- Lentiviral vectors are engineered to be replication incompetent so that the infection will not spread beyond the site of infection. However there is a risk of generating infective replication competent virus through recombination. Later generation vectors in which 3 or 4 plasmids are used to produce the viral particles and which a deletion is created in the LTR upon integration, have a lower risk of generating replication competent virus, so their use is encouraged where practical.
- Lentiviral vectors are generally classed as biohazard risk group 2. However they may be classed as risk group 2+ and require additional operational precautions if the vector system is an earlier generation, or if the transgene encodes a biological toxin, an oncogene, a cell cycle regulator or an inhibitor of a tumor suppressor (eg. siRNA for a tumor suppressor). If this is the case it will be indicated on the biohazard permit approved by the Queen's Biohazard Committee.
- Lentivirus cannot replicate in rodents, but because rodents can shed virus for days after infection, infection of rodents must occur at containment level 2. If the animals have been transplanted with human cells, then if replication competent virus is generated then it could replicate in the transplanted cells.

5. Biohazard Containment Facilities and Procedures:

- All work with lentivirus should be carried out in a containment level 2 laboratory. Lentivirus in supernatants and cells must be inactivated before removal to lower containment. Inactivation must be by an approved method (eg. 0.5% Triton-X 100 extraction of cells at 37°C for 30 min.).
- If cells are to be transported to another level 2 containment laboratory approved for lentiviral work, a secondary container with a tight fitting lid to prevent spills must be used for transport.
- If cells are infected that would normally only require level 1 containment, then the cells can be removed from level 2 containment after demonstrating that there is no longer any infective virus in the culture supernatant. This often requires three or four passages (not simply washing). Demonstration of the absence of lentivirus in the culture supernatant may be done using a p24 ELISA, with the supernatant from the day of infection used as a positive control

and from uninfected cells as a negative control. In addition to demonstrating the destruction of the input viruses, this is necessary to exclude the possible, although rare, development of a replication competent virus.

- Virus will be shed from rodents infected with lentivirus for a few days after infection, during which time the animals must be held at containment level 2. After the cage and bedding have been changed and shedding of virus has ceased (as demonstrated by an ELISA for p24 antigen or other approved method) then the animals may be moved to containment level 1. However, if the animals have been transplanted with human cells, then there is a risk that the virus could replicate in the transplanted cells and the animals must be handled at containment level 2.
- Unless higher containment practices are required by the Queen's Biohazard Committee, strict biosafety level 2 operational practices should be employed for manipulations of lentivirus. Review the Public Health Agency of Canada Laboratory Biosafety Guidelines, chapter 3 (3rd edition, 2004) before beginning work with lentivirus. **In particular, the following points are reinforced:**
  - Gloves should be worn during all tissue culture manipulations. Double gloves are better because micro-holes may be present. Inspect gloves for obvious holes when you are putting them on. Change outer gloves at regular intervals and whenever they become obviously contaminated. Wash your hands thoroughly with soap immediately after removing gloves. Lab coats with knit cuffs are recommended to ensure that no bare skin is exposed between the gloves and the lab coat.
  - Always treat your outer gloves as contaminated – remove them and replace with clean gloves before touching things outside of the biological safety cabinet (e.g. the microscope, the centrifuge, or the incubator); if necessary use the one clean hand / one dirty hand technique.
  - People often touch their face and eyes unconsciously. If you find that you are doing this with gloves on your hands then wear goggles to prevent yourself from doing so since this could result in you infecting yourself.
  - All work must be done in a biological safety cabinet so that the virus is not spread via aerosols. Use proper technique, not over-filling the cabinet and not putting anything on the front grill because this will disrupt the air flow, reducing your protection and potentially resulting in contamination of your cultures.
  - Centrifugation must take place in screw capped tubes (including microfuge tubes). Do not over-fill the tubes and do decontaminate their outer surface with 70% ethanol or quaternary ammonium disinfectant before removing them from the biological safety cabinet. Aerosol resistant centrifuge cups that are opened only in the biological safety cabinet are recommended, but not strictly required.
  - The garbage containing lentivirus contaminated dishes, filters, syringes, gloves etc. should be collected inside the biological safety cabinet. Immediately after completing work, seal it into a second autoclave bag and take it to the autoclave for disinfection. Unseal for autoclaving to allow steam penetration.

- Avoid the use of sharps whenever possible and dispose of them immediately in a sharps container within the biological safety cabinet. If feasible use plastic disposable transfer pipettes rather that glass Pasteur pipettes. Eliminate the use of needles whenever possible or use safety engineered needles. Use disposable scalpels and safety engineered scalpels.
- Glass serological pipettes and Pasteur pipettes should be immediately submerged into jars filled with freshly diluted bleach (1:10 dilution of bleach) inside of the biological safety cabinet. After 30 minutes in bleach, Pasture pipettes may be transferred to the glass disposal container and serological pipettes moved to a bucket for washing. If space constraints make it seem unreasonable to decontaminate serological pipettes in the biological safety cabinet, then discuss alternatives with the University Biosafety Officer.
- The plastic tubing and aspiration bottle should be disinfected immediately after use by drawing concentrated bleach through the line and into the collection bottle. Allow the bottle to sit for 30 minutes for full decontamination after the last addition before discarding the liquid in the sewer. After emptying the collection bottle add sufficient bleach that a 10% dilution will be achieved when the bottle is full. Be sure that the vacuum is protected with a HEPA filter.
- Liquid spills on any surfaces should be immediately disinfected with diluted bleach (30 minute contact time). Spills that are allowed to dry are much more difficult to decontaminate and must be rehydrated first. Be sure to rinse off the bleach well from stainless steal because it will corrode.
- The dishes containing lentivirus should be put in a secondary container to move from the biological safety cabinet to the incubator to reduce the risk of spills. They should be kept in a designated incubator whenever possible. Take care not to contaminate the door handle of the incubator.
- When finished work, decontaminate the outer surface of everything that is in the biological safety cabinet and then remove all items from the cabinet. Wash the hood thoroughly with 70% alcohol or other approved disinfectant. The use of UV light to disinfect the hood is not recommended because it is not effective unless properly maintained and because it can present a UV exposure hazard to other users of the laboratory unless the cabinet has a sash that can be completely closed.
- Ensure that the appended reminder sheet is posted in the laboratory where lentivirus is used.

6. First Aid:

- Following a splash or accidental touching of the eyes, nose or mouth with material potentially contaminated with lentivirus, **immediately** flush the eyes or other mucosal surfaces at an

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eyewash station for **15 minutes**. Remember to remove your gloves before using your fingers to keep your eyes open.

- Wear double gloves to protect broken skin. A wound or broken skin (eg. chapped, eczema) potentially contaminated with lentivirus should be **immediately** washed with soap and running water for 15 minutes with gentle massaging.
- Seek follow-up medical attention if required.
- Report the incident to your supervisor and have them fill in an incident report (WSIB form 7) which is to be submitted to the Department of Environmental Health and Safety within 24 hours of the incident.

7. **Information and Enquires:** University Biosafety Officer (613-533-6000 ext. 77077)

8. **Revision History:**

Initial release: March 2008

ATCC

MATERIAL SAFETY DATA SHEET

MSDS FOR ANIMAL CELL CULTURES (Biosafety Level 1 or 2)

ATCC cultures are not hazardous as defined by OSHA 1910.1200. However, as live cells they are potential biohazards.

ATCC Emergency Telephone: (703) 365-2710 (24 hours)
Chemtrec: (800) 424-9300
To be used only in the event of an emergency involving a spill, leak, fire, exposure or accident.

Description

Either frozen or growing cells shipped in liquid cell culture medium (a mixture of components that may include, but is not limited to: inorganic salts, vitamins, amino acids, carbohydrates and other nutrients dissolved in water).

SECTION I
Hazardous Ingredients

Frozen cultures may contain 5 to 10% Dimethyl sulfoxide (DMSO)

SECTION II
Physical data

Pink or red aqueous liquid

SECTION III
Health hazards

For Biosafety Level 1 Cell Lines

This cell line is not known to harbor an agent known to cause disease in healthy adult humans. This cell line has **NOT** been screened for Hepatitis B, human immunodeficiency viruses or other adventitious agents. Handle as a potentially biohazardous material under at least Biosafety Level 1 containment.

For Biosafety Level 2 Cell Lines

This cell line is known to contain an agent that requires handling at Biosafety Level 2 containment [U.S. Government Publication **Biosafety in Microbiological and Biomedical Laboratories** (CDC, 1999)]. These agents have been associated with human disease. This cell line has **NOT** been screened for Hepatitis B, human immunodeficiency viruses or other adventitious agents. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

SECTION IV
Fire and explosion

Not applicable

SECTION V
Reactivity data

Stable. Hazardous polymerization will not occur.

SECTION VI
Method of disposal

Spill: Contain the spill and decontaminate using suitable disinfectants such as chlorine bleach or 70% ethyl or isopropyl alcohol.

Waste disposal: Dispose of cultures and exposed materials by autoclaving at 121°C for 20 minutes. Follow all Federal, State and local regulations.

SECTION VII
Special protection information

For Biosafety Level 1 Cell Lines

Handle as a potentially biohazardous material under at least Biosafety Level 1 containment. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

For Biosafety Level 2 Cell Lines

Handle as a potentially biohazardous material under at least Biosafety Level 2 containment. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

SECTION VIII
Special precautions or comments

ATCC recommends that appropriate safety procedures be used when handling all cell lines, especially those derived from human or other primate material. Detailed discussions of laboratory safety procedures are provided in **Laboratory Safety: Principles and Practice** (Fleming, et al., 1995) the ATCC manual on quality control (Hay, et al., 1992), the Journal of Tissue Culture Methods (Caputo, 1988), and in the U.S. Government Publication, **Biosafety in Microbiological and Biomedical Laboratories** (CDC, 1999). This publication is available in its entirety in the Center for Disease Control Office of Health and Safety's web site at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

THE ABOVE INFORMATION IS CORRECT TO THE BEST OF OUR KNOWLEDGE. ALL MATERIALS AND MIXTURES MAY PRESENT UNKNOWN HAZARDS AND SHOULD BE USED WITH CAUTION. THE USER SHOULD MAKE INDEPENDENT DECISIONS REGARDING THE COMPLETENESS OF THE INFORMATION BASED ON ALL SOURCES AVAILABLE. ATCC SHALL NOT BE HELD LIABLE FOR ANY DAMAGE RESULTING FROM HANDLING OR CONTACT WITH THE ABOVE PRODUCT.

February 2002

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Cell Biology

ATCC® Number: CCL-34™ Order this Item
Price: $269.00

Designations: MDCK (NBL-2)

Depositors: S Madin, NB Darby

Biosafety Level: 1

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: *Canis familiaris*

Morphology: epithelial

PHOTO

Source: **Organ:** kidney
Disease: normal

Cellular Products: keratin

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC matenal. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Isolation: **Isolation date:** September, 1958

Applications: transfection host (Nucleofection technology from Lonza
Roche FuGENE® Transfection Reagents)

Virus Susceptibility:
Human Coxsackievirus B 5
Reovirus type 2
Adeno-associated virus 4
Vaccinia virus
Vesicular stomatitis virus
Adeno-associated virus 5
Human Coxsackievirus B 3
Human Coxsackievirus B 4
Human poliovirus 2

Reverse Transcript: negative

Cytogenetic Analysis: Polyploidy 0.2%. Two large submetacentric chromosomes noted, presumably X chromosomes, and one or two additional chromosomes with median or submedian centromeres.

Age: adult

Gender: female

Comments: The MDCK cell line was derived from a kidney of an apparently normal adult female cocker spaniel, September, 1958, by S.H. Madin and N.B. Darby.The cells are positive for keratin by immunoperoxidase staining.MDCK cells have been used to study processing of beta amyloid precursor protein and sorting of its proteolytic products.

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Cell Biology

ATCC® Number: CL-101™ Order this Item
Price: $275.00

Designations: LLC-PK1
Depositors: Eli Lilly & Co.
Biosafety Level: 1
Shipped: frozen
Medium & Serum: See Propagation
Organism: Sus scrofa (pig)
Morphology: epithelial
Source: **Organ:** kidney
Strain: Hampshire
Disease: normal
Cellular Products: plasminogen activator
Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Age: 3 to 4 weeks
Gender: male
Propagation: **ATCC complete growth medium:** The base medium for this cell line is Medium 199 containing 1.5 g/L sodium bicarbonate. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 3%.
Temperature: 37.0°C
Subculturing: **Subcultivation Ratio:** A subcultivation ratio of 1:3 to 1:8 is recommended
Medium Renewal: Twice per week
Remove medium, and rinse with 0.25% trypsin, 0.03% EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37C) until the cells detach.
Add fresh culture medium, aspirate and dispense into new culture flasks.
Preservation: culture medium 95%; DMSO, 5%
Related Products: recommended serum:ATCC 30-2020
formerly distributed as:ATCC CRL-1392
References:
3520: Hull RN, Huseby RM. Enhanced production of plasminogen activator. US Patent 3,904,480 dated Sep 9 1975
22659: Perantoni A, Berman JJ. Properties of Wilms' tumor line (TuWi) and pig kidney line (LLC-PK1) typical of normal kidney tubular epithelium. In Vitro 15: 446-454, 1979. PubMed: 225262
28301: Loffler S, et al. CD9, a tetraspan transmembrane protein, renders cells susceptible to canine distemper virus. J. Virol. 71: 42-49, 1997. PubMed: 8985321

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Cell Biology

ATCC® Number: CRL-1573™ Order this Item
Price: $256.00

Designations: 293 [HEK-293]
Depositors: FL Graham
Biosafety Level: 2 [CELLS CONTAIN ADENOVIRUS]
Shipped: frozen
Medium & Serum: See Propagation
Growth Properties: adherent
Organism: *Homo sapiens* (human)
Morphology: epithelial

PHOTO

Source:
Organ: embryonic kidney
Cell Type: transformed with adenovirus 5 DNA

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Restrictions: These cells are distributed for research purposes only. 293 cells, their products, or their derivatives may not be distributed to third parties.

Applications:
efficacy testing [92587]
transfection host (Nucleofection technology from Lonza
Roche FuGENE® Transfection Reagents)
viruscide testing [92579]

Receptors: vitronectin, expressed

Tumorigenic: Yes

DNA Profile (STR):
Amelogenin: X
CSF1PO: 11,12
D13S317: 12,14
D16S539: 9,13
D5S818: 8,9
D7S820: 11,12
THO1: 7,9.3
TPOX: 11
vWA: 16,19

Cytogenetic Analysis: This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %. The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired. There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells.

Age: fetus

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Cell Biology

ATCC® Number:	CRL-11268™ Order this Item
Designations:	293T/17 [HEK 293T/17]
Depositors:	Rockefeller Univ.
Biosafety Level:	2 [Cells contain Adeno and SV-40 viral DNA sequences]
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial
Source:	**Organ:** kidney
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Restrictions:	The line is available with the following restriction: 1. The cell line was deposited at the ATCC by Rockefeller University and is provided for research purposes only. Neither the cell line nor the products derived from it may be sold or used for commercial purposes. Nor can the cells be distributed to third parties for purposes of sale, or producing for sale, cells or their products. The cells are provided as a service to the research community. They are provided without warranty of merchantability or fitness for a particular purpose or any other warranty, expressed or implied. 2. Any proposed commercial use of the cells, or their products, must first be negotiated with Cell Genesys, 500 Forbes Boulevard, South San Francisco, CA 94080 Attn: Robert H. Tidwell; Senior Vice President, Corporate Development.
Antigen Expression:	SV40 T antigen [45408]
Age:	fetus
Comments:	The 293T/17 cell line is a derivative of the 293T (293tsA1609neo) cell line. 293T is a highly transfectable derivative of the 293 cell line into which the temperature sensitive gene for SV40 T-antigen was inserted. 293T cells were cloned and the clones tested with the pBND and pZAP vectors to obtain a line capable of producing high titers of infectious retrovirus, 293T/17. These cells constitutively express the simian virus 40 (SV40) large T antigen, and clone 17 was selected specifically for its high transfectability.293T/17 cells were cotransfected with the pCRIPenv- and the pCRIPgag-2 vectors to obtain the ANJOU 65 (see ATCC CRL-11269) cell line.ANJOU 65 cells were cotransfected with the pCRIPgag-2 and pGPT2E vectors to obtain the BOSC 23 (see ATCC CRL-11270) ecotropic envelope-expression packaging cell line.ANJOU 65 cells were also cotransfected with the pCRIPAMgag vector along with a plasmid expressing the gpt resistance gene to obtain the Bing (see ATCC CRL-11554) amphotropic envelope-expression packaging cell line.

Price: $272.00

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ATCC® Number: TIB-152™ Order this Item **Price:** $272.00

Designations:	Jurkat, Clone E6-1
Depositors:	A Weiss
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	suspension
Organism:	*Homo sapiens* (human)
Morphology:	lymphoblast
	PHOTO
Source:	**Disease:** acute T cell leukemia **Cell Type:** T lymphocyte;
Cellular Products:	interleukin-2 (interleukin 2, IL-2) [1609]
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)
Receptors:	T cell antigen receptor, expressed
Antigen Expression:	CD3; Homo sapiens, expressed
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 11,12 D13S317: 8,12 D16S539: 11 D5S818: 9 D7S820: 8,12 THO1: 6,9.3 TPOX: 8,10 vWA: 18
Cytogenetic Analysis:	This is a pseudodiploid human cell line. The modal chromosome number is 46, occurring in 74% with polyploidy at 5.3%. The karyotype is 46,XY,-2,-13,del(2) (p21p23),del(18) (p11.2). Most cells had normal X and Y chromosomes.
Gender:	male

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ATCC® Number:	CRL-11904™ Order this Item	**Price:**	$289.00
Designations:	JAWSII		
Depositors:	ZymoGenetics, Inc.		
Biosafety Level:	1		
Shipped:	frozen		
Medium & Serum:	See Propagation		
Growth Properties:	mixed, adherent and suspension		
Organism:	*Mus musculus* (mouse)		
Morphology:	monocyte PHOTO		
Source:	**Organ:** bone marrow **Strain:** C57BL/6 **Cell Type:** immature dendritic cell; monocyte;		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Propagation:	**ATCC complete growth medium:** Alpha minimum essential medium with ribonucleosides, deoxyribonucleosides, 4 mM L-glutamine, 1 mM sodium pyruvate and 5 ng/ml murine GM-CSF, 80%; fetal bovine serum, 20% **Temperature:** 37.0°C		
Subculturing:	**Protocol:** Cultures can be maintained by transferring floating cells to a centrifuge tube. Attached cells may be subcultured using 0.25% trypsin-0.03% EDTA. Pool cells and centrifuge the cell suspension at 1000 rpm for 10 minutes, resuspend the pellet in fresh medium, aspirate and dispense into new flasks. Note: This cell line grows very slowly. **Subcultivation Ratio:** A subcultivation ratio of 1:2 is recommended **Medium Renewal:** Once per week		
Preservation:	**Freeze medium:** Complete growth medium 95%; DMSO, 5% **Storage temperature:** liquid nitrogen vapor phase		
Related Products:	recommended serum:ATCC 30-2020		
References:	38868: MacKay VL, Moore EE. Immortalized dendritic cells. US Patent 5,648,219 dated Jul 15 1997 47440: Moore EE. Preparation of immortalized cells. US Patent 5,830,682 dated Nov 3 1998		

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Cell Biology

ATCC® Number:	**CRL-2190™** Order this Item
Designations:	HK-2
Depositors:	RA Zager
Biosafety Level:	2 [Cells Contain Papilloma viral DNA sequences]
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial
Source:	**Organ:** kidney, cortex **Tissue:** proximal tubule **Cell Type:** human papillomavirus 16 (HPV-16) transformed
Cellular Products:	alkaline phosphatase; gamma glutamyltranspeptidase; leucine aminopeptidase; acid phosphatase; cytokeratin; alpha 3, beta 1 integrin; fibronectin
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Receptors:	epidermal growth factor (EGF), expressed
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 13 D13S317: 9 D16S539: 11,12 D5S818: 12 D7S820: 10,11 THO1: 9 TPOX: 8,9 vWA: 17,18
Age:	adult
Gender:	male

Price: $272.00

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Cell Biology

ATCC® Number: CRL-2123™ Order this Item
Price: $349.00

Designations: mIMCD-3

Depositors: S Gullans

Biosafety Level: 2 [CELLS CONTAIN PAPOVAVIRUS]

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: Mus musculus, transgenic (mouse, transgenic)

Morphology: epithelial

Source:
Organ: kidney, medulla
Tissue: collecting duct
Cell Type: SV40 transformed

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Isolation: **Isolation date:** 1991

Applications: transfection host (Roche FuGENE® Transfection Reagents)

Age: adult

Comments: mIMCD-3 is an inner medullary collecting duct (IMCD) cell line derived in 1991 by Michael Rauchman from a mouse transgenic for the early region of SV40 [Tg(SV40E)bn/7].
A tubule from the terminal one-third of the IMCD was microdissected and placed in culture.
Confluent cells were subcultured and cloned using cloning cylinders.
This is a polarized epithelia cell line which retains many differentiated characteristics of the terminal IMCD including inhibition of apical to basal sodium flux by amiloride and by atrial natriuretic peptide (ANP).
The cells possess an amiloride sensitive sodium channel as determined by Western blot analysis, and accumulate the major organic osmolytes (inositol, sorbitol, betaine and glycerophosphorylcholine) in response to hypertonic stress.
The cells secrete endothelin and form tubules and tight junctions.
mIMCD-3 cells are responsive to Hepatocyte Growth Factor (HGF), and are readily adaptable to growth in hypertonic medium supplemented with NaCl and urea up to 910 mosmol/kg H20.
These extreme osmotic conditions exist in the renal medulla in vivo, but are known to be lethal to most other cells.

Propagation: **ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated DMEM:F12 Medium Catalog No. 30-2006. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Temperature: 37.0°C
Atmosphere: air, 95%; carbon dioxide (CO2), 5%

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Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for A

ATCC® Number:	**CRL-1932™** Order this Item **P**
Designations:	786-O [786-0]
Depositors:	RD Williams
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial
Source:	**Organ:** kidney **Disease:** renal cell adenocarcinoma
Cellular Products:	parathyroid hormone (PTH) like peptide
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulat permits may be required for the transfer of this ATCC material. Any purchasing ATCC material is ultimately responsible for obtaining permits. Please click here for information regarding the spe requirements for shipment to your location.
Applications:	transfection host (technology from amaxa)
Tumorigenic:	Yes
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 10 D13S317: 8 D16S539: 12 D5S818: 9 D7S820: 11,12 THO1: 6,9.3 TPOX: 8,11 vWA: 15,17
Cytogenetic Analysis:	hypertriploid; Y was present in 60% the cells examined
Age:	58 years
Gender:	male
Ethnicity:	Caucasian

Product Description

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ATCC® Number:	**CCL-223™** Order this Item	**Price:**	**$329.00**
Designations:	CMT-93		**Related Links**
Depositors:	LM Franks		NCBI Entrez Search
Biosafety Level:	1		Make a Deposit
Shipped:	frozen		Frequently Asked Questions
Medium & Serum:	See Propagation		Material Transfer Agreement
Growth Properties:	adherent		Technical Support
Organism:	*Mus musculus* (mouse)		Related Cell Culture Products
Morphology:	epithelial		
Source:	**Strain:** C57BL/icrf **Organ:** rectum **Disease:** polyploid carcinoma		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Tumorigenic:	Yes		
Reverse Transcript:	positive		
Antigen Expression:	H-2b		
GenoType:	a(t)		
Age:	19 months		
Gender:	male		
Comments:	Tested and found negative for ectromelia virus (mousepox).		
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0°C		

SANTA CRUZ BIOTECHNOLOGY, INC.

TIM-1 siRNA (h): sc-61691

BACKGROUND

CD4+ T helper lymphocytes can be divided into types 1 (Th1) and 2 (Th2) on the basis of their cytokine secretion patterns. Th1 cells and their associated cytokines are involved in cell-mediated immunity to intracellular pathogens and delayed-type hypersensitivity reactions. Th2 cells are involved in the control of extracellular helminthic infections and the promotion of atopic and allergic diseases. T cell Ig- and mucin-domain-containing molecules (TIMs) are a family of molecules expressed on T cells. TIM-1 is a single-pass type I membrane protein that is associated with the development of Th2 biased immune responses and selectively expressed on Th2 cells. TIM-1, also designated hepatitis A virus cellular receptor-1 (HAVcr-1) or T cell membrane protein 1, acts as a cell-surface receptor for hepatitis A virus and may also play a role in asthma and allergic disease regulation. TIM-1 is a widely expressed protein with highest levels detected in testis and kidney.

REFERENCES

1. Feigelstock, D., et al. 1998. The human homolog of HAVcr-1 codes for a hepatitis A virus cellular receptor. J. Virol. 72: 6621-6628.
2. McIntire, J.J., et al. 2003. Immunology: hepatitis A virus link to atopic disease. Nature 425: 576.
3. de Souza, A.J., et al. 2005. T cell Ig and Mucin 1 (TIM-1) is expressed on *in vivo*-activated T cells and provides a costimulatory signal for T cell activation. Proc. Natl. Acad. Sci. USA 102: 17113-17118.
4. Mariat, C., et al. 2005. Regulation of T cell dependent immune responses by TIM family members. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 360: 1681-1685.

CHROMOSOMAL LOCATION

Genetic locus: HAVCR1 (human) mapping to 5q33.2.

PRODUCT

TIM-1 siRNA (h) is a pool of 3 target-specific 19-25 nt siRNAs designed to knock down gene expression. Each vial contains 3 nmol of lyophilized siRNA, sufficient for a 10 µM solution once resuspended using protocol below. Suitable for 50-100 transfections. Also see TIM-1 shRNA Plasmid (h): sc-61691-SH and TIM-1 shRNA (h) Lentiviral Particles: sc-61691-V as alternate gene silencing products.

For independent verification of TIM-1 (h) gene silencing results, we also provide the individual siRNA duplex components. Each is available as 3 nmol of lyophilized siRNA. These include: sc-61691A, sc-61691B and sc-61691C.

STORAGE AND RESUSPENSION

Store lyophilized siRNA duplex at -20° C with desiccant. Stable for at least one year from the date of shipment. Once resuspended, store at -20° C, avoid contact with RNAses and repeated freeze thaw cycles.

Resuspend lyophilized siRNA duplex in 330 µl of the RNAse-free water provided. Resuspension of the siRNA duplex in 330 µl of RNAse-free water makes a 10 µM solution in a 10 µM Tris-HCL, pH 8.0, 20 mM NaCl, 1 mM EDTA buffered solution.

APPLICATIONS

TIM-1 siRNA (h) is recommended for the inhibition of TIM-1 expression in human cells.

SUPPORT REAGENTS

For optimal siRNA transfection efficiency, Santa Cruz Biotechnology's siRNA Transfection Reagent: sc-29528 (0.3 ml), siRNA Transfection Medium: sc-36868 (20 ml) and siRNA Dilution Buffer: sc-29527 (1.5 ml) are recommended. Control siRNAs or Fluorescein Conjugated Control siRNAs are available as 10 µM in 60 µl. Each contain a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA. Fluorescein Conjugated Control siRNAs include: sc-36869, sc-44239, sc-44240 and sc-44241. Control siRNAs include: sc-37007, sc-44230, sc-44231, sc-44232, sc-44233, sc-44234, sc-44235, sc-44236, sc-44237 and sc-44238.

GENE EXPRESSION MONITORING

TIM-1 (BBCR): sc-80359 is recommended as a control antibody for monitoring of TIM-1 gene expression knockdown by Western Blotting (starting dilution 1:200, dilution range 1:100-1:1000) or immunofluorescence (starting dilution 1:50, dilution range 1:50-1:500).

To ensure optimal results, the following support (secondary) reagents are recommended: 1) Western Blotting: use goat anti-mouse IgG-HRP: sc-2005 (dilution range: 1:2000-1:32,000) or Cruz Marker™ compatible goat anti-mouse IgG-HRP: sc-2031 (dilution range: 1:2000-1:5000), Cruz Marker™ Molecular Weight Standards: sc-2035, TBS Blotto A Blocking Reagent: sc-2333 and Western Blotting Luminol Reagent: sc-2048. 2) Immunofluorescence: use goat anti-mouse IgG-FITC: sc-2010 (dilution range: 1:100-1:400) or goat anti-mouse IgG-TR: sc-2781 (dilution range: 1:100-1:400) with UltraCruz™ Mounting Medium: sc-24941.

RT-PCR REAGENTS

Semi-quantitative RT-PCR may be performed to monitor TIM-1 gene expression knockdown using RT-PCR Primer: TIM-1 (h)-PR: sc-61691-PR (20 µl). Annealing temperature for the primers should be 55-60° C and the extension temperature should be 68-72° C.

RESEARCH USE

For research use only, not for use in diagnostic procedures.

PROTOCOLS

See our web site at www.scbt.com or our catalog for detailed protocols and support products.

Lentiviral Expression System

Visit our website for more details! click here

Obtain high-level expression in virtually any cell type with our complete Lenti-X™ Expression System

- **Optimized lentiviral vector and packaging system for high titers and high expression**
- **Transfer genes into dividing and nondividing cells and stem cells**
- **Puromycin resistance allows rapid selection of transduced cells**
- **Safe, replication-incompetent virus**

Recombinant lentiviruses derived from HIV-1 are able to deliver genes into almost any mammalian cell type, including primary cultures, dividing or nondividing cells, and stem cells. Clontech has developed a highly advanced lentiviral expression system that provides the broad cellular tropism of VSV-G pseudotyped lentivirus, high viral titers, and excellent transgene expression. The **Lenti-X Expression System,** which includes the **pLVX-Puro** expression vector and our **Lenti-X HT Packaging System,** enables you to produce exceptionally high titers of safe, replication-incompetent lentivirus from your customized pLVX-Puro vector (Figure 1).

Superior Lenti-X Vectors

Like all our Lenti-X vectors, pLVX-Puro not only carries the LTRs and packaging sequence required for lentivirus production and replication, but it also contains elements that improve transgene expression, titer, and overall vector function. Its WPRE element, believed to promote RNA processing events and nuclear export, imparts a dual benefit (1). First, it acts within the context of viral genomic transcripts to enhance vector packaging and increase the titers of viral supernatants produced from 293T packaging cells. Second, it boosts expression of your gene of interest in target cells by facilitating the production of mature mRNA from transcripts initiated by the vector's internal CMV promoter. Lenti-X vectors also contain a cPPT element that increases nuclear importation of the viral genome during target cell infection, resulting in improved vector integration and more efficient transduction (2).

High-Efficiency Packaging

Our Lenti-X HT Packaging System produces outstanding viral titers due to a synergism of highly optimized components (3). The Lenti-X HT Packaging Mix safely provides all the essential lentiviral packaging and replication gene products *in trans* on a proprietary suite of separate vectors. Selected plasmids in the mixture generate high expression levels for critical viral proteins as a result of Tet-Off® transactivation. For added safety, a split gag-pol gene delivery strategy thoroughly prevents viral replicative functions from being transferred to target cells (3). Finally, the included **Lentiphos™ HT** transfection reagents transfer the Lenti-X HT Packaging Mix, along with your pLVX-Puro vector, into 293T cells with unprecedented efficiency. The resulting high-titer viral supernatants can be used directly, without concentration.

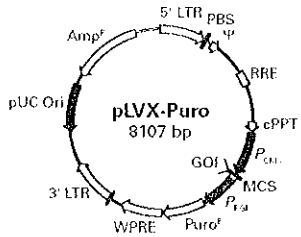


Figure 1. Map of pLVX-Puro. The vector contains the lentiviral-specific LTRs and packaging sequence (Ψ); a multiple cloning site (MCS) to insert your gene of interest (GOI); puromycin resistance; and WPRE and cPPT elements to boost packaging, viral titers, and transgene expression.

High Titers & Rapid Selection

We used the Lenti-X Expression System to generate a high-titer pLVX-Puro supernatant, serial dilutions of which were used to infect naïve cultures of 293T cells (Figure 2). After replating the infected cells on 10 cm dishes and selecting transductants with puromycin, the resulting colonies of stable transductants were stained for detection. Cells infected with only 0.1 µl of supernatant produced hundreds of colonies, while colonies from cells infected with 1 µl virtually covered the entire plate. These results demonstrate the high titer and infectivity of a typical pLVX-Puro supernatant.

Product	Size	Cat. No.	Price
Lenti-X Expression System	each	632164	$1,096.00
Puromycin	25 mg	631305	$71.00
	100 mg	631306	$176.00

Prices are subject to change without notice.

Components

- pLVX-Puro Vector
- Lenti-X™ HT Packaging Mix
- Lentiphos™ HT
- Lenti-X™ Lentiviral Expression Systems User Manual (PT3983-1)

Related Products

- Lenti-X™ Fluorescent Vectors (Cat. Nos. 632152, 632153, 632154 & 632155)
- Lenti-X™ HT Packaging System (Cat. Nos. 632160 & 632161)

Notice to Purchaser

Please see the bGH Poly A, CMV Sequence, cPPT Element, IRES Sequence, Lentiviral Expression Products, Tet-Based Expression Products, VSV-G Technology, and WPRE Technology licensing statements at **www.clontech.com/licensing**

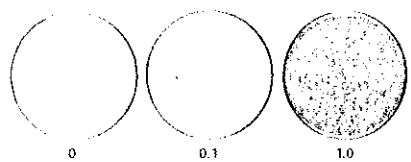


Figure 2. Puromycin selection of transduced cells. 293T cells were infected with the indicated volumes (µl) of pLVX-Puro supernatant and selected with puromycin for 9 days to allow the formation of colonies, which were then stained with crystal violet.

The Lenti-X Expression System is a comprehensive system for preparing recombinant lentivirus to express any cDNA in any cell type susceptible to lentivirus transduction. It easily produces high-titer lentiviral supernatants suitable for safe use with virtually any downstream application.

References

1. Zufferey, R. *et al.* (1999) *J. Virol.* 73(4):2886–2892.
2. Zennou, V. *et al.* (2000) *Cell* 101(2):173–185.
3. Wu, X. *et al.* (2000) *Mol. Ther.* 2(1):47–55.

DAY 1

- Plate target cells in a 12-well plate 24 hours prior to viral infection.
- Add 1 ml of complete optimal medium (with serum and antibiotics) and incubate cells overnight. The cells should be approximately 50% confluent on the day of infection (Day 2).

NOTE: It is possible to use other plate formats for transduction as well. In this case, the amount of cells should be adjusted depending on the growth area of the well or plate.

DAY 2

- Prepare a mixture of complete medium with Polybrene® (sc-134220) at a final concentration of 5 µg/ml.
- Remove media from plate wells and replace with 1 ml of this Polybrene/media mixture per well (for 12-well plate).

NOTE: Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined (usually in the range of 2-10 µg/ml). Excessive exposure to Polybrene (>12 hr) can be toxic to some cells.

- Thaw lentiviral particles at room temperature and mix gently before use.
- Infect cells by adding the shRNA Lentiviral Particles to the culture.
- Swirl the plate gently to mix and incubate overnight. The amount of viral particles to use varies greatly depending on the characteristics of the cell line used.

NOTE: Keep thawed shRNA Lentiviral Particles on ice. Repeated freeze-thaw cycles and prolonged exposure of the particles to ambient temperatures may result in decreased viral titers.

NOTE: When transducing a shRNA lentiviral construct into a cell for the first time we suggest using several amounts of shRNA lentiviral particle stock. In addition, we recommend to include one well with cells transduced with Control shRNA Lentiviral Particles (sc-108080).

DAY 3

- Remove the culture medium and replace with 1 ml of complete medium (without Polybrene).
- Incubate the cells overnight.

DAY 4

- To select stable clones expressing the shRNA, split cells 1:3 to 1:5, depending on the cell type, and continue incubating for 24-48 hours in complete medium.

DAY 5-6 and forward

- Select stable clones expressing the shRNA via Puromycin dihydrochloride (sc-108071) selection.
- For puromycin selection, use an amount sufficient to kill the non-transduced cells. Puromycin concentrations ranging from 2 to 10 µg/ml are usually sufficient, but a puromycin tritation is recommended when using a new cell line.
- Replace medium with fresh puromycin-containing medium every 3-4 days, until resistant colonies can be identified. Pick several colonies, expand them and assay them for stable shRNA expression.

NOTE: Resulting puromycin-resistant clones may have varying levels of shRNA expression due to the random integration of the lentiviral construct into the genome of the cell.

NOTE: For shRNA expression analysis by Western Blot, prepare cell lysate as follows:

- Wash cells once with PBS.
- Lyse cells in 100 µl of a 1:1 mixture of 2x Electrophoresis Sample Buffer (sc-24945) and RIPA Lysis Buffer (sc-24948) by gently rocking the 12-well plate or by pipetting up and down.
- Sonicate the lysate on ice if necessary.

NOTE: For shRNA expression analysis by RT-PCR, isolate RNA using the method described by P. Chomczynski and N. Sacchi (1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159) or a commercially available RNA isolation kit.

BIOSAFETY

Lentiviral particles can be employed in standard Biosafety Level 2 tissue culture facilities (and should be treated with the same level of caution as with any other potentially infectious reagent). Lentiviral particles are replication-incompetent and are designed to self-inactivate after transduction and integration of shRNA constructs into genomic DNA of target cells.

shRNA LENTIVIRAL PARTICLES SUPPORT REAGENTS

PRODUCT	CAT. #	DESCRIPTION	AMOUNT
Control shRNA Lentiviral Particles	sc-108080	Control shRNA Lentiviral Particles is available as an alternate negative scrambled shRNA sequence control.	200 µl
copGFP Control Lentiviral Particles	sc-108084	copGFP Control Lentiviral Particles are provided as transduction-ready viral particles.	10-20 transductions
Electrophoresis Sample Buffer	sc-24945	Ready-to-use reducing electrophoresis sample buffer solution for the preparation of protein samples to be separated by SDS-PAGE.	25 ml; 2X concentrate
RIPA Lysis Buffer	sc-24948	For use in mammalian cell lysis, with protease inhibitors. Available in four vials: 1X Lysis buffer, PMSF, protease inhibitor cocktail and sodium orthovanadate.	50 ml
Puromycin dihydrochloride	sc-108071	Available for selection and maintenance of cells transfected with the puromycin-N-acetyl-transferase (pac) gene.	25 mg
Polybrene®	sc-134220	Highly efficient infection reagent used to introduce retroviral vectors into mammalian cells.	1 ml

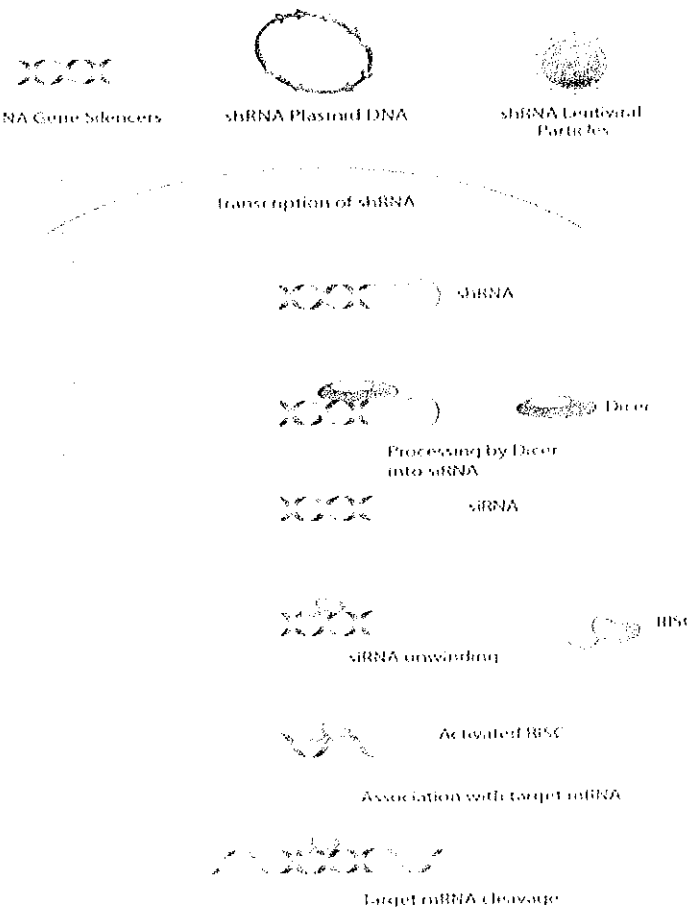
RNAi Gene Silencers

RNA interference (RNAi) was first identified in C. elegans by Nobel laureates Fire and Mello (1), and now represents one of the most promising discoveries in molecular biology. Endogenous RNAi activity has been linked to the regulation of transposon mobility (2), the determination of gene expression profiles (3) and cell fate (4), and is a crucial component of the innate cellular defense against viral infection in vivo(5). Three unique RNAi mechanisms controlling target gene expression have been demonstrated. RNAi regulates gene transcription by modifying heterochromatin formation (6). RNAi exercises two forms of post-transcriptional control. First RNAi can inhibit the translation of target mRNA (7) and second, RNAi can direct target mRNA destruction through the RISC complex (8). DICER first processes dsRNA leaving a two nucleotide long 3' overhang. This primes the dsRNA for binding to the RISC complex and leads to the activation of the enzyme activity of argonaute, the RNAse component of the RISC complex that destroys one of the RNA strands. The remaining guide strand, through complementary binding, then leads the RISC complex to associate with and cleave target RNA molecules.

The discovery of RNAi introduced an extraordinarily powerful laboratory tool for researchers and became a promising potential therapeutic tool, consequently leading to the 2006 Nobel Prize in Physiology or Medicine being awarded to Andrew Z. Fire and Craig C. Mello. In the laboratory, RNAi molecules are being used to downregulate individual target gene expression in a variety of organisms and cell types, exploiting each of the three mechanisms of inhibiting gene expression described above. These techniques are useful for manipulating an experimental system to explore individual gene and protein functions as well as their relationships to other genes and proteins. RNAi also has exciting clinical potential (9).

Details of these RNAi mechanisms are popular subjects of rigorous study, though much remains to be clarified. RNAi control of target mRNA degradation through the RISC complex, however, is the most well-described as well as the intended mechanism for RNAi Gene Silencers.

RNAi-directed mRNA Cleavage



Santa Cruz Biotechnology, Inc. offers a complete line of RNAi Gene Silencers, including siRNA, shRNA Plasmid and shRNA Lentiviral products covering > 99% of human and mouse protein encoding genes.

Click to jump to the section of your choice:

siRNA Gene Silencers shRNA Plasmids shRNA Lentiviral Particles Frequently Asked Questions Back to top

RNA interference Products offered by Santa Cruz Biotechnology Inc.

siRNA description:

- siRNA refers to small interfering or short interfering RNA
- Requires transfection of cells using a lipid-based transfection reagent
- Useful for a transient knock-down

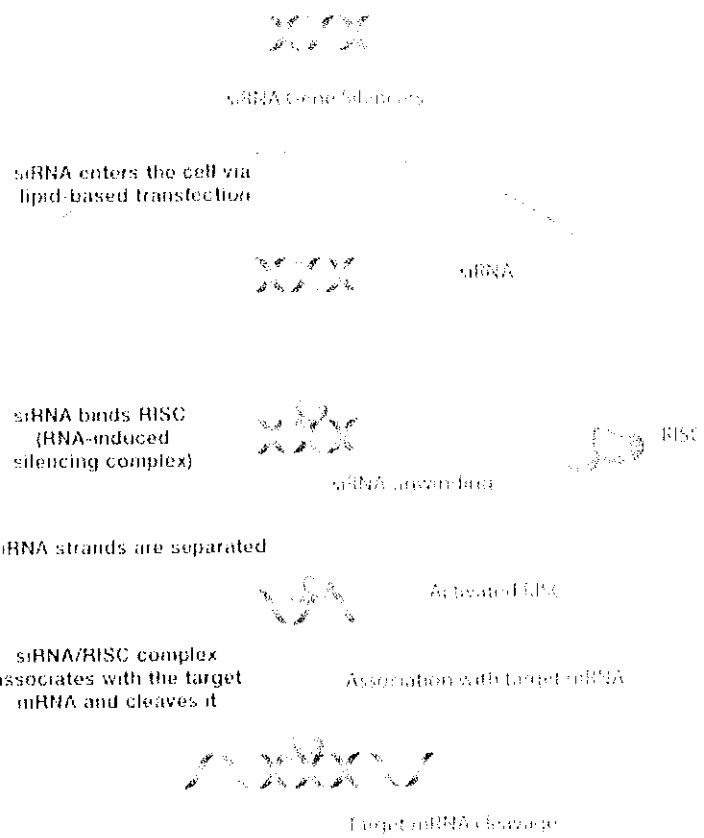
siRNA product details:

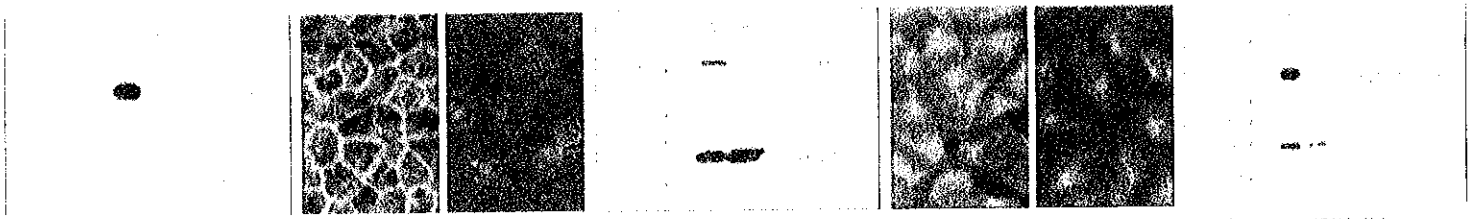
- siRNA Gene Silencers are pools of three target specific 19-25 nucleotide-long double stranded RNA molecules with 2-nt 3' overhangs on each end
- 10 µM, 50-100 transfections
- for independent verification of target gene silencing results, individual siRNA duplex components are also available upon request

Support Products for siRNA Gene Silencers:

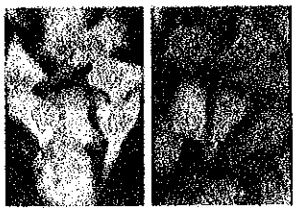
- suitable control antibodies are available
- RT-PCR Primers are available
- siRNA Dilution Buffer, sc-29527
- siRNA Transfection Reagent, sc-29528
- siRNA Transfection Medium, sc-36868
- siRNA Reagent System, sc-45064
- Control siRNAs, including Control siRNA-A, sc-37007
- Control siRNA (FITC Conjugate)-A, sc-36869

How do siRNA Gene Silencers work?





FGF-19 siRNA (h): sc-39480 | CD9 siRNA (h): sc-35032 | Daxx siRNA (h): sc-35178 | Cdc6 siRNA (h): sc-29258 | cytochrome c siRNA (h): sc-29292



cPLA2 siRNA (h): sc-29280 ERK 1 siRNA (m): sc-29308 c-Src siRNA (h): sc-29228 p53 siRNA (h): sc-29435 Lamin A/C siRNA (h): sc-35776

Click to jump to the section of your choice:

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shRNA Plasmid description:

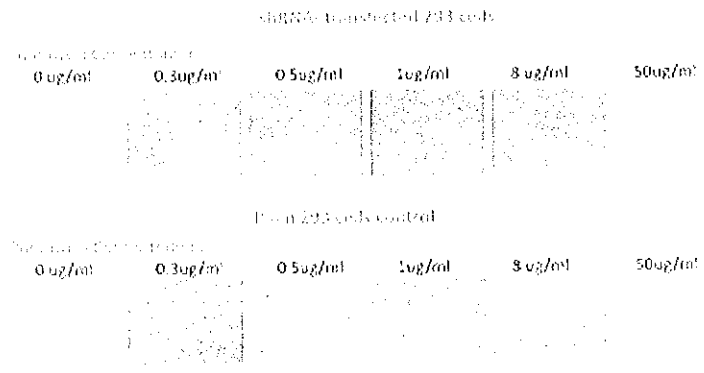
- shRNA refers to small hairpin or short hairpin RNA
- Plasmids encoding shRNA enter the cell via lipid-based transfection
- shRNA plasmids are capable of transient or stable inhibition of target gene expression
- shRNA Plasmids are provided as a pool of three to five lentiviral vector plasmids which each encode a target specific 19-25 nt shRNA with a 6 bp loop
- 20 µg, up to 20 transfections
- shRNA transcription is under the control of the H1 promoter
- provided as transfection-ready purified plasmid DNA
- After transfection, cells stably expressing shRNA can be selected by puromycin treatment

Support Products for shRNA Plasmid Gene Silencers:

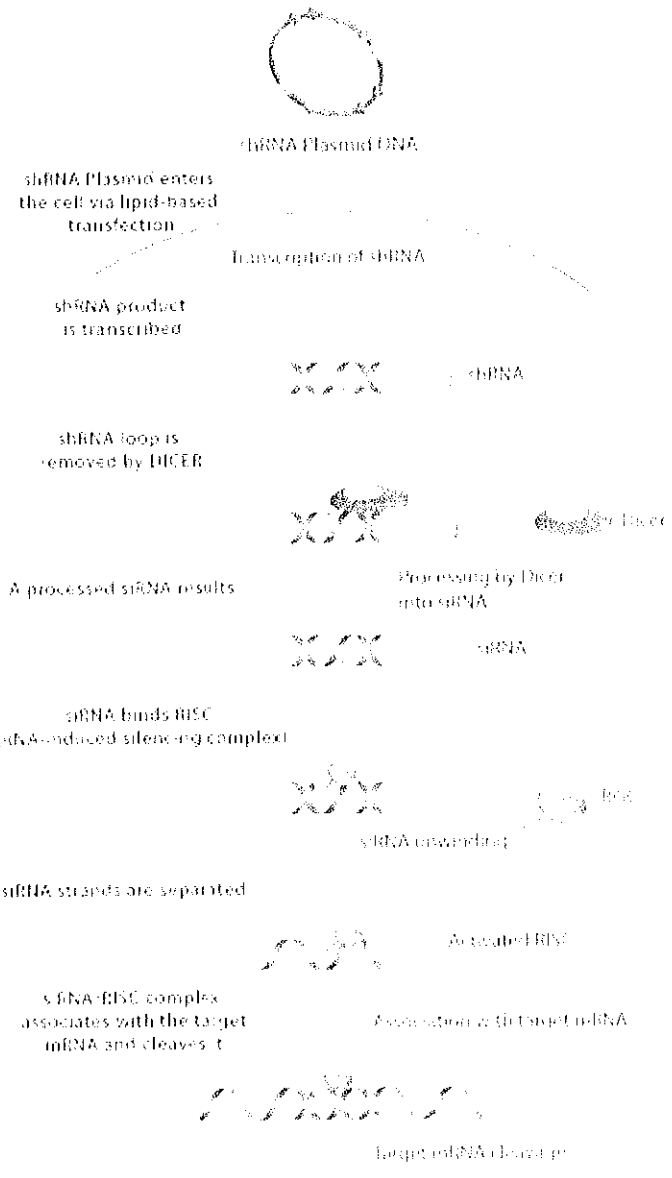
- suitable control antibodies are available
- RT-PCR Primers are available
- shRNA Plasmid Transfection Reagent, sc-108061
- shRNA Plasmid Transfection Medium, sc-108062
- Control shRNA Plasmid-A, sc-108060
- Control shRNA Plasmid-B, sc-108065
- Control shRNA Plasmid-C, sc-108066

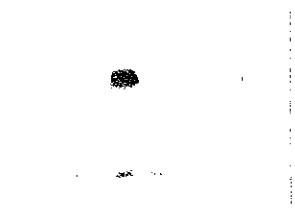
Confirm shRNA Plasmid Gene Silencer transfection efficiency with copGFP Control Plasmid: sc-108083

Generate Cells with stable expression of shRNA

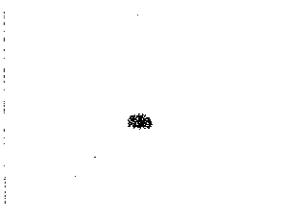


How do shRNA Plasmid Gene Silencers work?



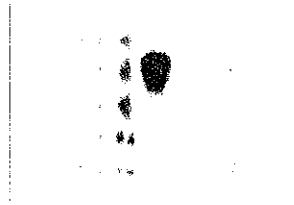


IL-1α shRNA Plasmid (h):
sc-39613-SH

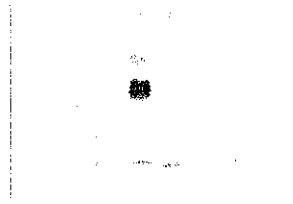


PTN shRNA Plasmid (m):
sc-39714-SH

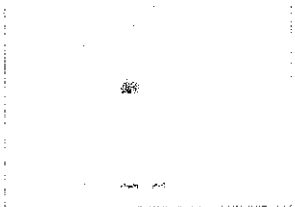
TCF-4 shRNA Plasmid (h):
sc-43525-SH



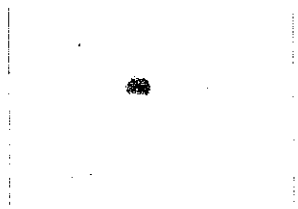
MIS shRNA Plasmid (h):
sc-39793-SH



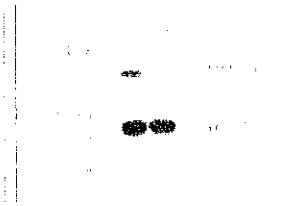
VEGF-D shRNA Plasmid (h):
sc-39844-SH



Amylase shRNA Plasmid (h):
sc-29675-SH



FGF-19 shRNA Plasmid (h):
sc-39480-SH



MMP-9 shRNA Plasmid (h):
sc-29400-SH

BMP-4 shRNA Plasmid (h):
sc-39744-SH

Cyr61 shRNA Plasmid (h):
sc-39331-SH

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How do Lentiviral Particle Gene Silencers work?

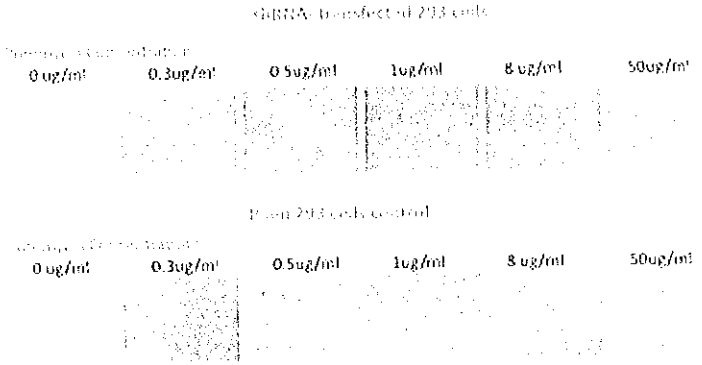
shRNA Lentiviral Particle description:

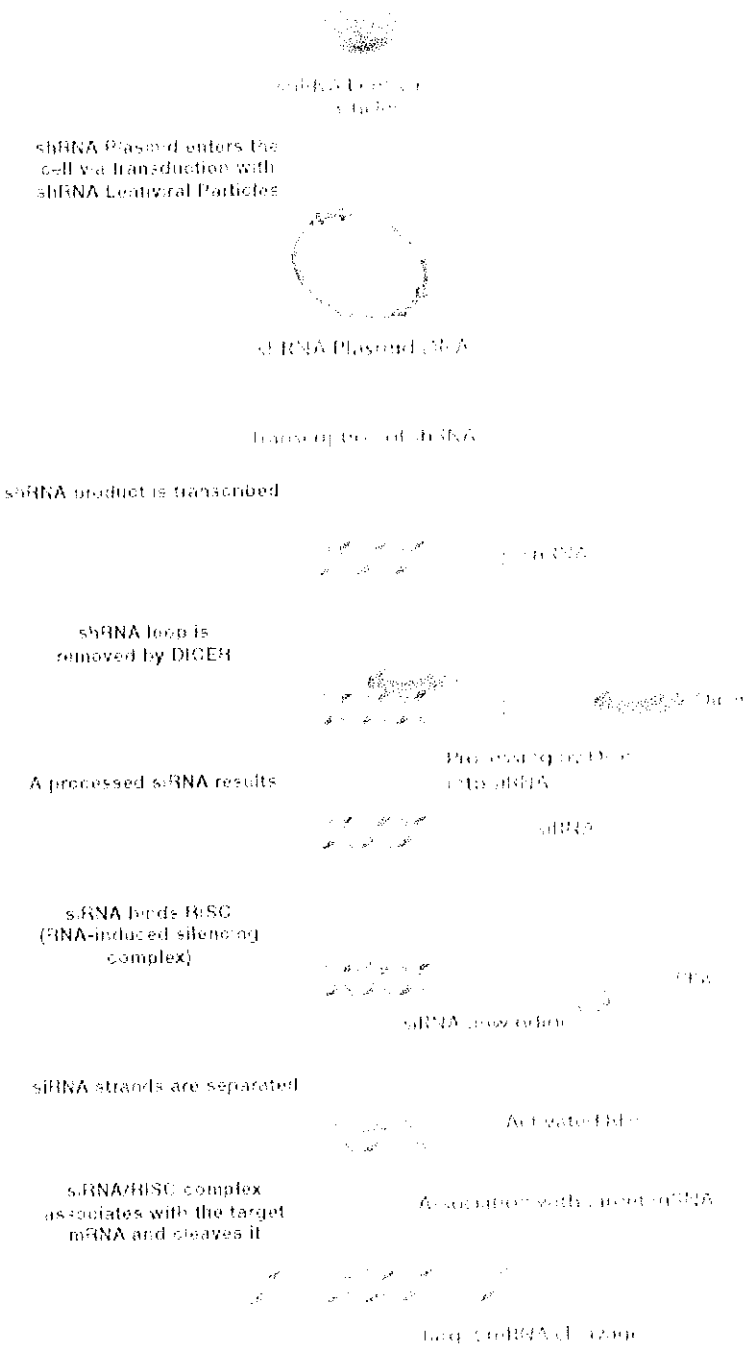
- shRNA refers to small hairpin or short hairpin RNA
- Lentiviral Particles deliver a shRNA encoding plasmid to target cell
- Useful for either transient or stable knock-down of a target gene
- Lentiviral Particles are provided as transduction-ready viruses for targeted gene silencing in mammalian cells (human or mouse)
- 200 µl viral stock containing 10^6 infectious lentiviral transducing particles per ml, sufficient for 10-20 transductions
- The Lentiviral Particles generally contain three to five expression constructs, each construct encoding a target specific 19-25 nt shRNA with a 6 bp loop
- After transduction, cells stably expressing shRNA can be selected by puromycin treatment
- copGFP Control Lentiviral Particles allow confirmation of the transduction efficiency of the Lentiviral Particles in a target cell population by expression of GFP detectable by either flow cytometry or fluorescence microscopy.
- The benefits of using shRNA Lentiviral Particles include avoiding harsh transfection techniques and the ability to introduce shRNA to any cell type
- Biosafety information - Lentiviral Particles are replication-incompetent and are designed to self-inactivate after transduction and integration of shRNA constructs into the genomic DNA of target cells.

Support Products for shRNA Lentiviral Particle Gene Silencers:

- suitable control antibodies are available
- RT-PCR Primers are available
- Control shRNA Lentiviral Particles: sc-108080
- copGFP Control Lentiviral Particles: sc-108084
- Puromycin dihydrochloride: sc-108071

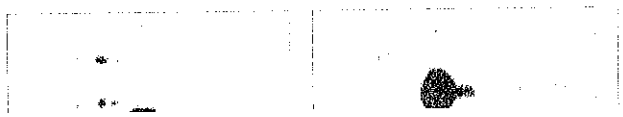
Generate Cells with stable expression of shRNA

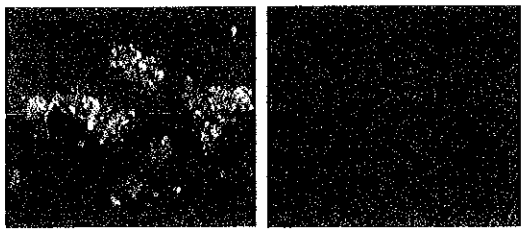




Use an effective Transduction Control

copGFP Control Lentiviral Particles





293T cells stably transduced with copGFP Control Lentiviral Particles (sc-108084) compared with non-transduced 293T cells as a negative control.

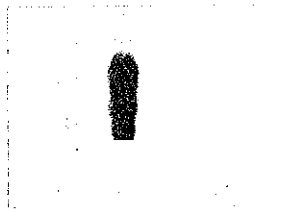
PNP shRNA (h) Lentiviral Particles: sc-45991-V

ephrin-A1 shRNA (m) Lentiviral Particles: sc-39427-V

MCP-4 shRNA Plasmid (h): sc-72122-SH

TNFβ shRNA Plasmid (h): sc-37218-SH

Somatostatin shRNA (h) Lentiviral Particles: sc-39728-V



Fos B shRNA (h) Lentiviral Particles: sc-35403-V

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Frequently Asked Questions:

What are the advantages of using shRNA versus siRNA?
Transfection of siRNA Gene Silencers into cultured cells provides a fast and efficient, though short-term, decrease in target gene expression. One may achieve stable gene silencing using shRNA Plasmids or shRNA Lentiviral Particles followed by puromycin selection. So, if one is targeting the expression of a protein with slow turnover, shRNA Plasmid or shRNA Lentiviral Particles would be ideal for accomplishing the goal.

What is the difference between using shRNA Lentiviral Particles versus shRNA Plasmids?
Transfection is required to use shRNA Plasmids for target gene silencing. Whereas shRNA Lentiviral Particles arrive ready to add to virtually any mammalian cell type, including primary and non-dividing cells. Both shRNA Plasmids and shRNA Lentiviral Particles may be used to develop stable expression of the shRNA with puromycin treatment. Lentiviral particles are shipped on dry ice while shRNA Plasmids are shipped on blue ice.

Do Lentiviral shRNA products pose any safety concerns?
Lentiviral particles can be employed in standard Biosafety Level 2 tissue culture facilities (and should be treated with the same level of caution as with any other potentially infectious reagent). The Lentiviral Particles are replication-incompetent and are designed to self-inactivate after transduction and integration of the shRNA constructs into the genomic DNA of target cells.

Are the sequences of your shRNA products the same as those for your related siRNA products to the same gene? Do you make those sequences available?
Yes. The sequences encoded in our shRNA Plasmids are the same as those used in the corresponding siRNA Gene Silencer products. These sequences are available to customers. Contact your Technical Service Representative.

The shRNA Plasmids are provided as a pool of three to five plasmids. Are they provided in separate vials? Are the individual shRNA plasmids of a pooled product sold separately?
The shRNA Plasmid products are provided in one vial. We offer the siRNA strands separately upon request. We may offer the plasmids separately in the future.

What kind of lentiviral vector do you use? What is the "vector name"?
The lentiviral vector we use is a custom made, proprietary vector. Please let us know what information you are looking for and why you need it. We might be able to answer your question without disclosing proprietary information.

What type of promoter does your vector use for shRNA transcription?
The vector uses a H1 promoter

What type of selection marker(s) are in the vector?
The vector has a Puromycin resistance gene encoding puromycin N-acetyltransferase enzyme for selection of successfully transfected or transduced cells.

How do you propagate the lentiviral vector plasmid?
The shRNA Plasmids and Lentiviral Particles are sold as transfection / transduction ready products. No additional preparation is necessary. shRNA Gene Silencers are consumable products for which no propagation protocols are provided.

What is copGFP and how is it helpful for use with the shRNA plasmids and Lentiviral Particles?
By administering the copGFP plasmid or copGFP Lentiviral Particles to a separate sample of target cells, one can identify the transfection or viral transduction efficiency for the target cell population. The copGFP plasmid and copGFP Lentiviral particles lead to expression of copepod green fluorescent protein which can be detected using a fluorescence microscope or flow cytometer.

What is the difference between (h) and (h2) shRNA products (for example E-Cadherin, sc-35242-SH and sc-44222-SH)?
The (h) and (h2) products are designed to silence the same gene, they have different sequences.

What support products and transfection reagents must I purchase from SCBT to use your shRNA Plasmids?
We recommend our shRNA Plasmid DNA Transfection Reagent, sc-108061 in addition to shRNA Plasmid DNA Transfection Medium, sc-108062. We also recommend our control shRNA Plasmid DNAs, either sc-108060 (A), sc-108065 (B) or sc-108066 (C). These encode scrambled shRNA sequences which will not target any known mammalian mRNA.

Click to jump to the section of your choice:

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Lentivirus Packaging and Production

The laboratories of Didier Trono (EPFL) and Robert Weinberg (Whitehead institute) have deposited plasmids for the production of lentiviral particles. These plasmids can be used with many lentiviral vectors, including The RNAi Consortium shRNA vectors being distributed by Sigma (i.e. MISSION shRNAs) and Open Biosystems (i.e. TRC shRNAs).

Overview

For producing lentiviral particles, you typically need three components: 1) a lentiviral vector, such as pLKO.1 or pLVTHM, containing the shRNA or transgene, 2) a packaging vector, such as psPAX2 or pCMV-dR8.2 dvpr, and 3) an envelope vector, such as pMD2.G or pCMV-VSVG.

For most applications, you can produce viral particles by transient transfection of 293T cells with a 2nd generation packaging system (e.g. packaging plasmid psPAX2 and envelope plasmid pMD2.G).

2nd Generation Packaging System

In general, lentiviral vectors with a wildtype 5' LTR need the 2nd generation packaging system because these vectors require TAT for activation. All lentiviral vectors from the Trono or Aebischer lab require packaging with a 2nd generation system.

Below are two 2nd generation systems. Lentiviral plasmids based on pLKO.1 can be packaged with either system, although the first system has been reported to produce higher titer. See Addgene's pLKO.1 Protocol for producing lentiviral particles.

2nd generation system deposited by the Trono lab:

ID	Plasmid	Description
12260	psPAX2	2nd generation packaging plasmid for producing viral particles. psPAX2 contains a robust CAG promoter for efficient expression of packaging proteins. Trono lab and Aebischer lab lentiviral vectors require psPAX2. Produces higher titer than pCMV-dR8.2 dvpr.
12259	pMD2.G	Envelope plasmid for producing viral particles

2nd generation system deposited by the Weinberg lab:

ID	Plasmid	Description
8455	pCMV-dR8.2 dvpr	2nd generation packaging plasmid for producing viral particles
8454	pCMV-VSVG	Envelope plasmid for producing viral particles

3rd Generation Packaging System

The 3rd generation packaging system offers maximal biosafety but is more cumbersome to use, as it involves the transfection of four different plasmids in the producer cells (two packaging plasmids, an envelope plasmid, and the lentiviral vector).

If you wish to use this system, you need to have a lentiviral vector with a chimeric 5' LTR in which the HIV promoter is replaced with CMV or RSV, thus making it TAT-independent. Examples of these vectors include pLKO.1, pLL3.7, pLB, p_enti6, pSico/pSicoR, pCL, pCS, and pLove.

Most Aebischer and Trono Lab lentiviral vectors CANNOT be used with this system. A lentiviral vector carrying a chimeric 5' LTR can be packaged with either the 2nd or 3rd generation packaging system.

ID	Plasmid	Description
12251	pMDLg/pRRE	3rd generation packaging plasmid for producing viral particles

12253	pRSV-Rev	3rd generation packaging plasmid for producing viral particles
12259	pMD2.G	Envelope plasmid for producing viral particles

More information

- Click here to browse other RNAi vectors, or search for plasmids using the search bar at the top of the page.
- Trono Lab website or Lentiweb: information and a discussion forum on cloning, packaging, and other protocols.
- Moffat J et. al. 2006. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell 124:1283-1298. (PubMed)
- Ventura et. al. 2004. Cre-lox-regulated conditional RNA interference from transgenes. PNAS 2004 Jul 13;101(28):10380-5. (PubMed)
- Naldini L et. al. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263-267. (PubMed)
- Dull et al., A Third-Generation Lentivirus Vector with a Conditional Packaging System. J. Virol. 1998 72(11): 8463-8472. (PubMed)
- Zufferey R et. al. 1997. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 15(9):871-5. (PubMed)
- Zufferey R et. al. 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 72(12):9873-80. (PubMed)

Cell Line

The 293T cell line for producing lentiviral particles can be obtained from GenHunter.

addgene

Search for Plasmids: Go

Advanced Search

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Plasmid Cart

Your cart is empty

Recently Viewed

pBABE-neo Plasmid 1767

Browse > Bob Weinberg > Article > pBABE-neo

Print Friendly Email

Price: $65.00

Plasmid 1767: pBABE-neo

	none
	Unknown
	pBABE-neo
(Search Vector Database)	Mammalian expression,Retroviral
	5330
pBABE 5' (List of Sequencing Primers)	
	pBABE 3'
	Ampicillin
	High Copy
	Yes
	Neomycin
View sequence	
View map	
	DH5a
	Bob Weinberg
pBABE protocol (PDF)	
MTA	

Author's map
Sequence
Reviews (0)

From this article
Bob Weinberg Lab Plasmids

pBABE-hygro
pBABE-puro
pBABE-zeo (pBABE-bleo)
pBABE GFP
pBABE-neo-hTERT

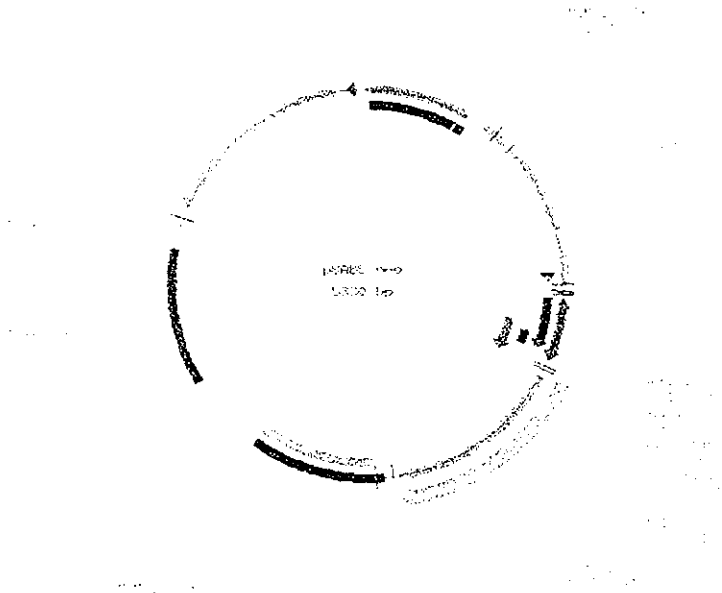
Morgenstern JP, Land H., 1990, Nucleic Acids Research 18(12):3587-96.

Note: There is an extra ~300 bp of vector sequence between the HindIII site and the neomycin gene that is not depicted in the author's sequence.

If you are using the pBABE protocol from the Weinberg Lab to generate virus, please note that Addgene supplies pCL-Eco (#12371), VSV-G (#8454), and a gag/pol expression vector (#8455).

Addgene has sequenced a portion of this plasmid for verification. Click here for the sequencing result.

Click on map to enlarge



5_LTR2	12 - 479	SpeI	616
3MoMuLV_LTR	18 - 479	AatII	701
psi_plus_pack	549 - 1350	BamHI	1355
pBABE_5_primer	1318 - 1334	EcoRI	1379
pBABE_3_primer	1428 - 1408	SalI	1397
SV40_enhancer	1629 - 1414	StuI	1726
SV40_promoter	1426 - 1694	HindIII	1743
SV40_origin	1593 - 1670	ClaI	2567
SV40_promoter	1546 - 1748	NheI	2641
SV40pro_F_primer	1655 - 1674	NotI	4294
ORF frame 3	1752 - 2564		
NeoR/KanR	1773 - 2561		
3MoMuLV_LTR	2611 - 3204		
5_LTR2	2653 - 3204		
pBR322_origin	4180 - 3561		
Ampicillin	5202 - 4342		
AmpR_promoter	5272 - 5244		

Please acknowledge the principal investigator if you use this plasmid in a publication.

Also, please include the text "Addgene plasmid 1767" in your Materials and Methods section.
This information allows Addgene to create a link from the plasmid page to your publication.

SIGMA-ALDRICH

Material Safety Data Sheet

Version 3.1
Revision Date 06/18/2009
Print Date 02/03/2010

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : ADP-ribosyltransferase C3, from *Clostridium botulinum*

Product Number : A8724
Brand : Sigma

Company : Sigma-Aldrich Canada, Ltd
2149 Winston Park Drive
OAKVILLE ON L6H 6J8
CANADA
Telephone : +19058299500
Fax : +19058299292
Emergency Phone # : 800-424-9300

2. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : Botulinum neurotoxin C3
C3 Exotoxin
C3 Transferase
C3 Exoenzyme

CAS-No.	EC-No.	Index-No.	Concentration
ADP-ribosyltransferase C3 from Clostridium botulinum			
58319-92-9	-	-	-

3. HAZARDS IDENTIFICATION

WHMIS Classification
D2B Toxic Material Causing Other Toxic Effects Moderate respiratory irritant

HMIS Classification
Health Hazard: 2
Flammability: 0
Physical hazards: 0

Potential Health Effects

Inhalation May be harmful if inhaled. Causes respiratory tract irritation.
Skin May be harmful if absorbed through skin. Causes skin irritation.
Eyes Causes eye irritation.
Ingestion May be harmful if swallowed.

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing give artificial respiration Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Consult a physician.

In case of eye contact
Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIRE-FIGHTING MEASURES

Flammable properties

Flash point	no data available
Ignition temperature	no data available

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use personal protective equipment. Avoid dust formation. Avoid breathing dust. Ensure adequate ventilation.

Environmental precautions
Do not let product enter drains.

Methods for cleaning up
Pick up and arrange disposal without creating dust. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Handling
Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: 2 - 8 °C

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
Where risk assessment shows air-purifying respirators are appropriate use a dust mask type N95 (US) or type P1 (EN 143) respirator. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves.

Eye protection
Safety glasses with side-shields conforming to EN166

Skin and body protection
Choose body protection according to the amount and concentration of the dangerous substance at the work place.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid

Safety data

pH	no data available
Melting point	no data available
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Water solubility	no data available

10. STABILITY AND REACTIVITY

Storage stability
Stable under recommended storage conditions.

Materials to avoid
Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Nature of decomposition products not known.

11. TOXICOLOGICAL INFORMATION

Acute toxicity

no data available

Irritation and corrosion

no data available

Sensitisation

Prolonged or repeated exposure may cause allergic reactions in certain sensitive individuals.

Chronic exposure

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

Signs and Symptoms of Exposure

Headache, Dizziness, To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Potential Health Effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.
Ingestion	May be harmful if swallowed.

12. ECOLOGICAL INFORMATION

Elimination information (persistence and degradability)

no data available

Ecotoxicity effects

no data available

Further information on ecology

no data available

13. DISPOSAL CONSIDERATIONS

Product

Observe all federal, state, and local environmental regulations. Contact a licensed professional waste disposal service to dispose of this material.

Contaminated packaging

Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)

Not dangerous goods

IMDG

Not dangerous goods

IATA

Not dangerous goods

15. REGULATORY INFORMATION

DSL Status

This product contains the following components that are not on the Canadian DSL nor NDSL lists.

	CAS-No.
ADP-ribosyltransferase C3 from Clostridium botulinum	58319-92-9

WHMIS Classification

D2B Toxic Material Causing Other Toxic Effects Moderate respiratory irritant

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Phalloidin, *Amanita phalloides*: sc-202763

The Power to Question

1. Identification of the substance/preparation and of the company/undertaking

Product name : **Phalloidin, *Amanita phalloides***

Chemical formula : $C_{35}H_{48}N_8O_{11}S$

Synonym : phalloidin

Catalog # : sc-202763

Supplier : Santa Cruz Biotechnology, Inc.
2145 Delaware Avenue
Santa Cruz, California 95060
800.457.3801 or 831.457.3800

2. Composition / information on ingredients

Substance/ Preparation : Substance

Chemical name	CAS No.	EC Number	Symbol	R-Phrases
Phalloidin *Amanita phalloides*	17466-45-4	241-484-5	T+	R27/28

3. Hazards identification

Physical chemical hazards : Not applicable

Human health hazards : DANGER!
MAY BE FATAL IF ABSORBED THROUGH SKIN OR IF SWALLOWED
MAY CAUSE DAMAGE TO THE FOLLOWING ORGANS: KIDNEYS, LIVER, GASTROINTESTINAL TRACT, CENTRAL NERVOUS SYSTEM.

4. First-aid measures

First-Aid measures

Inhalation : If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion : If swallowed, do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. Loosen tight clothing such as a collar, tie, belt or waistband. Get medical attention immediately.

Skin Contact : In case of contact, immediately flush skin with plenty of water. Remove contaminated clothing and shoes. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention.

Eye Contact : Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Most important symptoms

Ingestion : Extremely hazardous in case of ingestion. May be fatal if swallowed.

Skin Contact : Extremely hazardous in case of skin contact (permeator). Severe over-exposure can result in death.

Aggravating conditions : Repeated exposure to a highly toxic material may produce general deterioration of health by an accumulation in one or many human organs.

5. Fire-fighting measures

Flammability of the Product : May be combustible at high temperature.

Extinguishing Media

Suitable : SMALL FIRE: Use DRY chemical powder.
LARGE FIRE: Use water spray, fog or foam. Do not use water jet.

Hazardous thermal decomposition products : These products are carbon oxides (CO, CO2), nitrogen oxides (NO, NO2, ...), sulfur oxides (SO2, SO3, ...).

Special fire-fighting procedures : Fire fighters should wear positive pressure self-contained breathing apparatus (SCBA) and full turnout gear.

Protection of fire-fighters : Be sure to use an approved/certified respirator or equivalent.

6. Accidental release measures

Personal precautions	:	Splash goggles. Full suit. Dust respirator. Boots. Gloves. A self-contained breathing apparatus should be used to avoid inhalation of the product. Suggested protective clothing might not be sufficient; consult a specialist BEFORE handling this product.
Small Spill and Leak	:	Use appropriate tools to put the spilled solid in a convenient waste disposal container.
Large Spill and Leak	:	Stop leak if without risk. Do not get water inside container. Do not touch spilled material. Use water spray to reduce vapors. Prevent entry into sewers, basements or confined areas; dike if needed. Eliminate all ignition sources. Call for assistance on disposal.

7. Handling and storage

Handling	:	Keep locked up. Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not ingest. Do not breathe dust. Avoid contact with skin. Wear suitable protective clothing. If ingested, seek medical advice immediately and show the container or the label.
Storage	:	Keep container tightly closed. Keep container in a cool, well-ventilated area. Do not store above 4°C (39.2°F).
Packaging Materials		
Recommended	:	Use original container.

8. Exposure controls/personal protection

Engineering measures	:	Use process enclosures, local exhaust ventilation, or other engineering controls to keep airborne levels below recommended exposure limits. If user operations generate dust, fume or mist, use ventilation to keep exposure to airborne contaminants below the exposure limit.
Hygiene measures	:	Wash hands, forearms, and face thoroughly after handling compounds and before eating, smoking, using lavatory, and at the end of day.

Ingredient Name	Occupational Exposure Limits
Phalloidin, *Amanita phalloides*	Not available.

Personal protective equipment

Respiratory system	:	Dust respirator. Be sure to use an approved/certified respirator or equivalent.
Skin and body	:	Lab coat.
Hands	:	Gloves.
Eyes	:	Safety glasses
Protective Clothing - Pictograms	:	

9. Physical and chemical properties

Physical state	:	Solid.
Color	:	Colorless
Molecular Weight	:	788.9 g/mole
Melting Point	:	280 to 282°C (536 to 539.6°F)
Solubility	:	Easily soluble in methanol
Flash point	:	Not available
Explosive properties	:	Risks of explosion of the product in presence of mechanical impact: Not available Risks of explosion of the product in presence of static discharge: Not available

10. Stability and reactivity

Stability	:	The product is stable.
Conditions to avoid	:	Not available
Hazardous Decomposition Products	:	These products are carbon oxides (CO, CO2), nitrogen oxides (NO, NO2, ...), sulfur oxides (SO2, SO3, ...)

11. Toxicological information

RTECS	:	SE9800000
Local effects		
Skin irritation	:	Not available.
Acute toxicity	:	LD50: Not available. LC50: Not available.
Chronic toxicity	:	Repeated exposure to a highly toxic material may produce general deterioration of health by an accumulation in one or many human organs.
Other Toxic Effects on Humans	:	Toxic for humans or animal life. Extremely hazardous in case of skin contact (permeation) of ingestion.
		Not available.
		Not available
Teratogenic effects	:	Not available.
Mutagenic effects	:	Not available
Reproduction toxicity	:	Not available.
Teratogenic effects	:	Not available.

12. Ecological information

Ecotoxicity	:	Not available
Toxicity of the products of biodegradation	:	The products of degradation are less toxic than the product itself

13. Disposal considerations

Methods of disposal: Waste of residues/ Contaminated packaging	:	Waste must be disposed of in accordance with federal, state and local environmental control regulations

14. Transport information

In accordance with transport regulations		
UN number	:	UN2811
Land - Road/Railway		
Proper shipping name	:	Toxic solid, organic, n.o.s.*
ADR/RID Class	:	CLASS 6.1 Toxic substance.
Packing Group	:	II
ADR/RID label	:	
Sea		
Proper shipping name	:	Toxic solid, organic, n.o.s.*
IMDG Class	:	CLASS 6.1 Toxic substance
Packing Group	:	II
IMDG label	:	
Air		
Proper shipping name		Toxic solid, organic, n.o.s.*
IATA Number	:	UN2811
IATA/ICAO Class	:	CLASS 6.1 Toxic substance.

Packing Group : I

UN Number :

Special Provisions for Transport : Not available

15. Regulatory information

Regulations

Hazard symbols :

Classification : Very toxic

Risk Phrases : R27/28- Very toxic in contact with skin and if swallowed.

Safety Phrases : S22- Do not breathe dust.
S36/37/39- Wear suitable protective clothing, gloves and eye/face protection
S45- In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible)

Contains : - Phalloidin, *Amanita phalloides*

U.S. Federal Regulations : TSCA. No products were found.
SARA 302/304/311/312 extremely hazardous substances: No products were found.
SARA 302/304 emergency planning and notification: No products were found.
SARA 302/304/311/312 hazardous chemicals: No products were found.
SARA 311/312 MSDS distribution - chemical inventory - hazard identification: No products were found
SARA 313 toxic chemical notification and release reporting: No products were found
Clean Water Act (CWA) 307: No products were found.
Clean Water Act (CWA) 311: No products were found.
Clean air act (CAA) 112 accidental release prevention: No products were found.
Clean air act (CAA) 112 regulated flammable substances: No products were found
Clean air act (CAA) 112 regulated toxic substances: No products were found.

DSCL classification : CLASS: Highly toxic.
CLASS: Target organ effects

State Regulations :

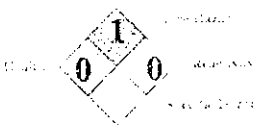
WHMIS (Canada) : Not controlled under WHMIS (Canada)
No products were found

16. Other information

Hazardous Material Information System (U.S.A.)

[illegible]	* 0
[illegible]	1
Reactivity	0
Personal Protection	E

National Fire Protection Association (U.S.A.)



The above information is believed to be correct but does not purport to be complete and should be used only as a guide. The burden of safe use of this material rests entirely with the user.

Emergency Contact:

Santa Cruz Biotechnology, Inc.
2145 Delaware Avenue
Santa Cruz, California 95060
800.457.3801 or 831.457.3800
or Luis Yanez 831.251.2179

ATCC

MATERIAL SAFETY DATA SHEET

SECTION 1 - SUBSTANCE IDENTITY AND COMPANY INFORMATION

Product Name: G418 Sulfate
ATCC Catalog No.: 30-2305

COMPANY INFORMATION: AMERICAN TYPE CULTURE COLLECTION
PO BOX 1549
MANASSAS, VA 20108
FOR INFORMATION CALL: 800-638-6597 or 703-365-2700
AFTER-HOURS CONTACT: 703-365-2710

CHEMTREC EMERGENCY: 800-424-9300 or 703-527-3887

SECTION 2 - COMPOSITION/INFORMATION ON INGREDIENTS

HAZARDOUS INGREDIENTS	CAS NUMBER	EC NUMBER (EINECS)	
G418	108321-42-2	UNLISTED	

SECTION 3 - HAZARDS IDENTIFICATION

EMERGENCY OVERVIEW:
HARMFUL BY INHALATION AND IF SWALLOWED.
AVOID CONTACT WITH SKIN AND EYES.
DO NOT BREATHE DUST.

WHEN USING, DO NOT EAT, DRINK, OR SMOKE.
IN CASE OF CONTACT WITH EYES, RINSE IMMEDIATELY WITH PLENTY OF WATER AND SEEK MEDICAL ADVICE.
WEAR SUITABLE PROTECTIVE CLOTHING AND GLOVES.
IN CASE OF ACCIDENT OR IF YOU FEEL UNWELL, SEEK MEDICAL ADVICE IMMEDIATELY (SHOW LABEL WHERE POSSIBLE).

TARGET ORGAN:
KIDNEYS.
EARS.
EYES.

SECTION 4 - FIRST AID MEASURES

EYES: FLUSH WITH PLENTY OF WATER FOR AT LEAST 15 MINUTES. ASSURE ADEQUATE FLUSHING BY SEPARATING THE EYELIDS WITH FINGERS. CALL A PHYSICIAN.
SKIN: IMMEDIATELY WASH SKIN WITH SOAP AND PLENTY OF WATER.
INGESTION: WASH OUT MOUTH WITH WATER PROVIDED PERSON IS CONSCIOUS. CALL A PHYSICIAN.
INHALATION: REMOVE TO FRESH AIR. IF BREATHING BECOMES DIFFICULT, CALL A PHYSICIAN.

SECTION 5 - FIRE FIGHTING MEASURES

EXTINGUISHING MEDIA:
WATER SPRAY, CARBON DIOXIDE, DRY CHEMICAL POWDER OR APPROPRIATE FOAM.

SPECIAL FIREFIGHTING PROCEDURES:
WEAR SELF-CONTAINED BREATHING APPARATUS AND PROTECTIVE CLOTHING TO PREVENT CONTACT WITH SKIN AND EYES.

UNUSUAL FIRE AND EXPLOSIONS HAZARDS:
EMITS TOXIC FUMES UNDER FIRE CONDITIONS. SUBSTANCE IS NONCOMBUSTIBLE.

SECTION 6 - ACCIDENTAL RELEASE MEASURES

WEAR SELF-CONTAINED BREATHING APPARATUS, RUBBER BOOTS AND RUBBER GLOVES.
WEAR DISPOSABLE COVERALLS AND DISCARD THEM AFTER USE.
SWEEP UP CAREFULLY TO AVOID CREATING AIRBORNE DUST.
PLACE IN A SUITABLE CONTAINER, SEAL, LABEL, AND HOLD FOR WASTE DISPOSAL.
VENTILATE AREA AND WASH SPILL SITE AFTER MATERIAL PICKUP IS COMPLETE. EVACUATE

SECTION 7 - HANDLING AND STORAGE

STORE AT REFRIGERATED TEMPERATURES (4 to 8° C). KEEP CONTAINER TIGHTLY CLOSED.

SECTION 8 - EXPOSURE CONTROLS/PERSONAL PROTECTION

MECHANICAL EXHAUST REQUIRED.
WEAR APPROPRIATE NIOSH/MSHA-APPROVED RESPIRATOR, CHEMICAL-RESISTANT GLOVES, SAFETY GOGGLES, AND OTHER PROTECTIVE CLOTHING.
EMERGENCY SHOWER AND EYE WASH STATION SHOULD BE READILY AVAILABLE.
AVOID CONTACT WITH EYES, SKIN AND CLOTHING.
AVOID PROLONGED OR REPEATED EXPOSURE.
WASH THOROUGHLY AFTER HANDLING.
WASH CONTAMINATED CLOTHING BEFORE REUSE.

SECTION 9 - PHYSICAL AND CHEMICAL PROPERTIES

APPEARANCE AND ODOR:

WHITE TO OFF-WHITE ODORLESS POWDER.

PHYSICAL PROPERTIES:
DATA NOT AVAILABLE.

SECTION 10 - STABILITY AND REACTIVITY

STABILITY:
STABLE.

INCOMPATIBILITIES:
STRONG OXIDIZING AGENTS.

HAZARDOUS COMBUSTION OR DECOMPOSITION PRODUCTS:
CARBON MONOXIDE, CARBON DIOXIDE, NITROGEN OXIDES, SULFUR OXIDES.

HAZARDOUS POLYMERIZATION:
WILL NOT OCCUR.

SECTION 11 - TOXICOLOGICAL INFORMATION

ACUTE EFFECTS:
MAY CAUSE SKIN IRRITATION.
MAY BE HARMFUL IF ABSORBED THROUGH THE SKIN.
MAY CAUSE EYE IRRITATION.
MAY BE HARMFUL IF INHALED.
MATERIAL MAY BE IRRITATING TO MUCOUS MEMBRANES AND UPPER RESPIRATORY TRACT.
MAY BE HARMFUL IF SWALLOWED.
TO THE BEST OF OUR KNOWLEDGE, THE CHEMICAL, PHYSICAL, AND TOXICOLOGICAL PROPERTIES HAVE NOT BEEN THOROUGHLY INVESTIGATED.

SECTION 12 - ECOLOGICAL INFORMATION

DATA NOT AVAILABLE.

SECTION 13 - DISPOSAL CONSIDERATIONS

CONTACT A LICENSED WASTE DISPOSAL SERVICE TO DISPOSE OF THIS MATERIAL.
OBSERVE ALL FEDERAL, STATE, AND LOCAL ENVIRONMENTAL REGULATIONS.

SECTION 14 - TRANSPORT INFORMATION

DATA NOT AVAILABLE.

SCHEDULE B NUMBER: 2941.90.6000

SECTION 15 - REGULATORY INFORMATION

EUROPEAN INFORMATION:
RISK PHRASES 20/22
HARMFUL BY INHALATION AND IF SWALLOWED.
SAFETY PHRASES 20/21, 22, 24/25, 26, 36/37, 45
WHEN USING, DO NOT EAT, DRINK, OR SMOKE.
DO NOT BREATHE DUST.
AVOID CONTACT WITH SKIN AND EYES.
IN CASE OF CONTACT WITH EYES, RINSE IMMEDIATELY WITH PLENTY OF WATER AND SEEK MEDICAL ADVICE.
WEAR SUITABLE PROTECTIVE CLOTHING AND GLOVES.
IN CASE OF ACCIDENT OR IF YOU FEEL UNWELL, SEEK MEDICAL ADVICE IMMEDIATELY (SHOW LABEL WHERE POSSIBLE).

SECTION 16 - OTHER INFORMATION

July 2003

ATCC

MATERIAL SAFETY DATA SHEET

SECTION 1. CHEMICAL IDENTIFICATION

Product Name: Dimethylsulfoxide (DMSO)
ATCC Catalog No.: 4-X

SECTION 2. COMPOSITION/INFORMATION ON INGREDIENTS

HAZARDOUS INGREDIENTS	CAS NUMBER	EC NUMBER (EINECS)	PERCENTAGE
DIMETHYLSULFOXIDE	67-68-5	200-664-3	99 – 100%

SECTION 3. HAZARDS IDENTIFICATION

LABEL PRECAUTIONARY STATEMENTS:
IRRITANT.
IRRITATING TO EYES, RESPIRATORY SYSTEM AND SKIN.
COMBUSTIBLE LIQUID.
READILY ABSORBED THROUGH SKIN.

TARGET ORGAN(S):
EYES.
SKIN.
DO NOT BREATHE VAPOR.
IN CASE OF CONTACT WITH EYES, RINSE IMMEDIATELY WITH PLENTY OF WATER AND SEEK MEDICAL ADVICE.
WEAR SUITABLE PROTECTIVE CLOTHING.
MOISTURE SENSITIVE.

SECTION 4. FIRST-AID MEASURES

IF SWALLOWED, WASH OUT MOUTH WITH WATER PROVIDED PERSON IS CONSCIOUS. CALL A PHYSICIAN.
IF INHALED, REMOVE TO FRESH AIR. IF NOT BREATHING GIVE ARTIFICIAL RESPIRATION. IF BREATHING IS DIFFICULT, GIVE OXYGEN.
IN CASE OF CONTACT, IMMEDIATELY WASH SKIN WITH SOAP AND COPIOUS AMOUNTS OF WATER.
IN CASE OF CONTACT, IMMEDIATELY FLUSH EYES WITH COPIOUS AMOUNTS OF WATER FOR AT LEAST 15 MINUTES.

SECTION 5. FIRE FIGHTING MEASURES

EXTINGUISHING MEDIA:
WATER SPRAY, CARBON DIOXIDE, DRY CHEMICAL POWDER OR APPROPRIATE FOAM.

SPECIAL FIREFIGHTING PROCEDURES:
WEAR SELF-CONTAINED BREATHING APPARATUS AND PROTECTIVE CLOTHING TO PREVENT CONTACT WITH SKIN AND EYES.

UNUSUAL FIRE AND EXPLOSIONS HAZARDS:
EMITS TOXIC FUMES UNDER FIRE CONDITIONS.
COMBUSTIBLE LIQUID.
METHYL SULFOXIDE (DMSO) UNDERGOES A VIOLENT EXOTHERMIC REACTION ON MIXING WITH COPPER WOOL AND TRICHLOROACETIC ACID. ON MIXING WITH POTASSIUM PERMANGANATE IT WILL FLASH INSTANTANEOUSLY. IT REACTS VIOLENTLY WITH: ACID HALIDES, CYANURIC CHLORIDE, SILICON TETRACHLORIDE, PHOSPHORUS TRICHLORIDE AND TRIOXIDE, THIONYL CHLORIDE, MAGNESIUM PERCHLORATE, SILVER FLUORIDE, METHYL BROMIDE, IODINE PENTAFLUORIDE, NITROGEN PERIODATE, DIBORANE, SODIUM HYDRIDE, PERCHLORIC AND PERIODIC ACIDS. WHEN HEATED ABOVE ITS BOILING POINT METHYL SULFOXIDE DEGRADES GIVING OFF FORMALDEHYDE, METHYL MERCAPTAN, AND SULFUR DIOXIDE.

SECTION 6. ACCIDENTAL RELEASE MEASURES

WEAR RESPIRATOR, CHEMICAL SAFETY GOGGLES, RUBBER BOOTS AND HEAVY RUBBER GLOVES.
ABSORB ON SAND OR VERMICULITE AND PLACE IN CLOSED CONTAINERS FOR DISPOSAL.
VENTILATE AREA AND WASH SPILL SITE AFTER MATERIAL PICKUP IS COMPLETE. EVACUATE AREA.
AVOID CONTAMINATING WATER SUPPLY.

SECTION 7. HANDLING AND STORAGE

REFER TO SECTION 8.

SECTION 8. EXPOSURE CONTROLS/PERSONAL PROTECTION

MECHANICAL EXHAUST REQUIRED.
SAFETY SHOWER AND EYE BATH.
WASH THOROUGHLY AFTER HANDLING.
KEEP TIGHTLY CLOSED.
KEEP AWAY FROM HEAT AND OPEN FLAME.
STORE IN A COOL DRY PLACE AT 2-8°C.
NIOSH/MSHA-APPROVED RESPIRATOR.
COMPATIBLE CHEMICAL-RESISTANT GLOVES.
CHEMICAL SAFETY GOGGLES.
DO NOT BREATHE VAPOR.
AVOID CONTACT WITH DMSO SOLUTIONS CONTAINING TOXIC MATERIALS OR MATERIALS WITH UNKNOWN TOXICOLOGICAL PROPERTIES. DIMETHYL SULFOXIDE IS READILY ABSORBED THROUGH SKIN AND MAY CARRY SUCH MATERIALS INTO THE BODY.
AVOID PROLONGED OR REPEATED EXPOSURE.

SECTION 9. PHYSICAL AND CHEMICAL PROPERTIES

APPEARANCE AND ODOR:
CLEAR, COLORLESS LIQUID.
HYGROSCOPIC.
GARLIC-LIKE ODOR.

BOILING POINT:	189°C
MELTING POINT:	18.4°C
FLASHPOINT:	188.60°F/87°C
EXPLOSION LIMITS IN AIR:	
UPPER	28.5 %
LOWER	2.6 %
VAPOR PRESSURE:	0.42 MMHG @ 20°C
SOLUBILITY:	
WATER -Z1076	
ALCOHOLS, ETHYL	
SPECIFIC GRAVITY:	1.1 G
VAPOR DENSITY:	2.7 G/L
FREEZING POINT:	18.5°C

SECTION 10. STABILITY AND REACTIVITY

STABILITY:
STABLE.

INCOMPATIBILITIES:
PROTECT FROM MOISTURE.
ACID CHLORIDES.
PHOSPHORUS HALIDES.
STRONG ACIDS.
STRONG OXIDIZING AGENTS.
STRONG REDUCING AGENTS.

HAZARDOUS COMBUSTION OR DECOMPOSITION PRODUCTS:
CARBON MONOXIDE.
CARBON DIOXIDE.
SULFUR OXIDES.
FORMALDEHYDE.
MERCAPTANS.

HAZARDOUS POLYMERIZATION:
WILL NOT OCCUR.

SECTION 11. TOXICOLOGICAL INFORMATION

ACUTE EFFECTS:
TO THE BEST OF OUR KNOWLEDGE, THE CHEMICAL, PHYSICAL, AND TOXICOLOGICAL PROPERTIES HAVE NOT BEEN THOROUGHLY INVESTIGATED.
CAUSES SKIN IRRITATION.
READILY ABSORBED THROUGH SKIN.
MAY BE HARMFUL IF ABSORBED THROUGH THE SKIN.
CAUSES EYE IRRITATION.
MATERIAL IS IRRITATING TO MUCOUS MEMBRANES AND UPPER RESPIRATORY TRACT

MAY BE HARMFUL IF INHALED.
MAY BE HARMFUL IF SWALLOWED.
LD.
LC.
AVOID CONTACT WITH DMSO SOLUTIONS CONTAINING TOXIC MATERIALS OR MATERIALS WITH UNKNOWN TOXICOLOGICAL PROPERTIES. DIMETHYL SULFOXIDE IS READILY ABSORBED THROUGH SKIN AND MAY CARRY SUCH MATERIALS INTO THE BODY.

CHRONIC EFFECTS.
TARGET ORGAN(S):
EYES.
SKIN.

RTECS #: PV6210000:
METHYL SULFOXIDE.

IRRITATION DATA:

SKN-RBT 10 MG/24H OPEN MLD	AIHAAP 23,95,1962
SKN-RBT 500 MG/24H MLD	85JCAE -,1044,1986
EYE-RBT 100 MG	TXAPA9 39,129,1977
EYE-RBT 500 MG/24H MLD	85JCAE -,1044,1986

TOXICITY DATA:

ORL-RAT LD50:14500 MG/KG	TXAPA9 15,74,1969
SKN-RAT LD50:40 GM/KG	ANYAA9 141,96,1967
IPR-RAT LD50:8200 MG/KG	FCTOD7 22,665,1984
SCU-RAT LD50:12 GM/KG	ARZNAD 14,1050,1964
IVN-RAT LD50:5360 MG/KG	TXAPA9 7,104,1965
UNR-RAT LD50:1300 MG/KG	NTIS** AD-A159-418
ORL-MUS LD50:7920 MG/KG	CHTPBA 3,10,1968
SKN-MUS LD50:50 GM/KG	ANYAA9 141,96,1967
IPR-MUS LD50:2500 MG/KG	RPTOAN 35,300,1972
SCU-MUS LD50:14 GM/KG	ANYAA9 141,96,1967
IVN-MUS LD50:3100 MG/KG	TXAPA9 15,74,1969
UNR-MUS LD50:12 GM/KG	USXXAM #4767763
ORL-DOG LD50:>10 GM/KG	ANYAA9 141,96,1967
IVN-DOG LD50:2500 MG/KG	CNCRA6 31,7,1963
ORL-CKN LD50:12 GM/KG	JPPMAB 15,688,1963
ORL-MAM LD50:21400 MG/KG	GISAAA 39(4),86,1974
ORL-BWD LD50:100 MG/KG	TXAPA9 21,315,1972

TARGET ORGAN DATA:
BEHAVIORAL (ALTERED SLEEP TIME).
LUNGS, THORAX OR RESPIRATION (DYSPNAE).
LUNGS, THORAX OR RESPIRATION (CYANOSIS).
GASTROINTESTINAL (NAUSEA OR VOMITING).
LIVER (JAUNDICE, OTHER OR UNCLASSIFIED).
BLOOD (OTHER CHANGES)

EFFECTS ON FERTILITY (PRE-IMPLANTATION MORTALITY).
EFFECTS ON EMBRYO OR FETUS (FETOTOXICITY).
SPECIFIC DEVELOPMENTAL ABNORMALITIES (MUSCULOSKELETAL SYSTEM).
ONLY SELECTED REGISTRY OF TOXIC EFFECTS OF CHEMICAL SUBSTANCES (RTECS) DATA IS PRESENTED HERE. SEE ACTUAL ENTRY IN RTECS FOR COMPLETE INFORMATION.

SECTION 12. ECOLOGICAL INFORMATION

DATA NOT YET AVAILABLE.

SECTION 13. DISPOSAL CONSIDERATIONS

CONTACT A LICENSED PROFESSIONAL WASTE DISPOSAL SERVICE TO DISPOSE OF THIS MATERIAL.
THIS COMBUSTIBLE MATERIAL MAY BE BURNED IN A CHEMICAL INCINERATOR EQUIPPED WITH AN AFTERBURNER AND SCRUBBER.
OBSERVE ALL FEDERAL, STATE AND LOCAL ENVIRONMENTAL REGULATIONS.

SECTION 14. TRANSPORT INFORMATION

THIS PRODUCT CONTAINS NO HAZARDOUS SUBSTANCES AS DEFINED BY THE DEPARTMENT OF TRANSPORTATION REGULATIONS, CODIFIED IN TITLE 49 CFR SECTION 171.8 AT REPORTABLE QUANTITIES ACCORDING TO TABLE 1 OF APPENDIX A OF 49 CFR §172.101.

SECTION 15. REGULATORY INFORMATION

EUROPEAN INFORMATION:
IRRITANT.
R 36/37/38
IRRITATING TO EYES, RESPIRATORY SYSTEM AND SKIN.
S 23
DO NOT BREATHE VAPOR.
S 26
IN CASE OF CONTACT WITH EYES, RINSE IMMEDIATELY WITH PLENTY OF WATER AND SEEK MEDICAL ADVICE.
S 36
WEAR SUITABLE PROTECTIVE CLOTHING.

REVIEWS, STANDARDS, AND REGULATIONS:
OEL=MAK.
OEL-GERMANY: NO MAK ESTABLISHED, JAN1999.
OEL-RUSSIA: STEL 20 MG/M3, JAN1993.
OEL-SWEDEN: TWA 50 PPM (150 MG/M3), KTV 150 PPM (500 MG/M3), SKIN, JAN1999.
OEL-SWITZERLAND: MAK-W 50 PPM (160 MG/M3), SKIN, JAN1999.
NOHS 1974: HZD 80564; NIS 11; TNF 476; NOS 25; TNE 22461.
NOES 1983: HZD 80564; NIS 29; TNF 3507; NOS 40; TNE 44947; TFE 16837.
EPA GENETOX PROGRAM 1988, POSITIVE: ASPERGILLUS-ANEUPLOIDY; S CEREVISIAE GENE CONVERSION.

EPA GENETOX PROGRAM 1988. NEGATIVE: SHE-CLONAL ASSAY: CELL TRANSFORM.-MOUSE EMBRYO.
EPA GENETOX PROGRAM 1988. NEGATIVE: CELL TRANSFORM.-RLV F344 RAT EMBRYO.
EPA GENETOX PROGRAM 1988. NEGATIVE: D MELANOGASTER-WHOLE SEX CHROM LOSS: HOST-MEDIATED ASSAY.
EPA GENETOX PROGRAM 1988. NEGATIVE: N CRASSA-ANEUPLOIDY: E COLI POLA WITH S9.
EPA GENETOX PROGRAM 1988. NEGATIVE: HISTIDINE REVERSION-AMES TEST: IN VITRO SCE-NONHUMAN.
EPA GENETOX PROGRAM 1988. NEGATIVE: D MELANOGASTER SEX-LINKED LETHAL.
EPA GENETOX PROGRAM 1988. INCONCLUSIVE: ASPERGILLUS-RECOMBINATION: CARCINOGENICITY-MOUSE/RAT.
EPA GENETOX PROGRAM 1988. INCONCLUSIVE: D MELANOGASTER-RECIPROCAL TRANSLOCATION.
EPA GENETOX PROGRAM 1988. INCONCLUSIVE: RODENT DOMINANT LETHAL: B SUBTILIS REC ASSAY.
EPA GENETOX PROGRAM 1988. INCONCLUSIVE: E COLI POLA WITHOUT S9 EPA TSCA SECTION 8(B) CHEMICAL INVENTORY.
EPA TSCA SECTION 8(D) UNPUBLISHED HEALTH/SAFETY STUDIES EPA TSCA TEST SUBMISSION (TSCATS) DATA BASE. JANUARY 2001.

SECTION 16. OTHER INFORMATION

THE ABOVE INFORMATION IS CORRECT TO THE BEST OF OUR KNOWLEDGE. ALL MATERIALS AND MIXTURES MAY PRESENT UNKNOWN HAZARDS AND SHOULD BE USED WITH CAUTION. THE USER SHOULD MAKE INDEPENDENT DECISIONS REGARDING THE COMPLETENESS OF THE INFORMATION BASED ON ALL SOURCES AVAILABLE. ATCC SHALL NOT BE HELD LIABLE FOR ANY DAMAGE RESULTING FROM HANDLING OR CONTACT WITH THE ABOVE PRODUCT.

March 2002